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# Detection of Cytocher cal and Morphological Anomalies in Preleul emia

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*Key Words:* Preleukemia · Sideroblastic nemia · Acute my logenous leukemia · Cytochemistry · Hemopoietic maturation defects

*Abstract.* The present investigations confirm and extend previous reports on the occurrence of cytochemical anomalies among the hemopoietic cells in preleukemia. The cytochemical patterns of the hemopoietic cells obtained from 19 patients suffer ing from preleukemic disorder have been studied. The diagnostic value of the cy tochemical examination using panel of 10 different tests is emphasized. In pro spective study in 17 out of 25 patients, preleukemia had been correctly diagnosed. In 4 patients leukemia developed within 2–4 months from the diagnosis ('imminent leukemia'), in 13 patients leukemia or smouldering leukemia developed between 4 and 25 months after the diagnosis ('true preleukemia'). The cytochemical profiles of the hemopoietic cells of the latter have been compared with those of 14 patients suffering from aplastic anemia. I preleukemia the blasts did not exceed 5% of the nucleated bone marrow cells. *The clinical value of the diagnosis 'preleukemia' is* discussed as well as the prognosis and the possibl therapeutic approaches.

The present investigations are based on two facts.

(1) In a considerable proportion of cases, the manifestation of acute myelogenous leukemia (AML) is preceded by disorders of the hemopoiet ic bone marrow without clear-cut evidence of the impending leukemic ac cumulation of blasts.

(2) The abnormal maturation of the hemopoietic cells is a characteristic finding in leukemia [9 36] and correspondingly a wide range of cytological anomalies has been detected in 'preleukemia by morphological, ultrastructural biochemical, cytochemical, and cytogenetic investigations [2, 4–8, 10, 15 19 26, 29–31 34 42, 46–48]

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The aim of our prospective study was to investigate whether the routine examination of blood and/or bone marrow smears by a panel of cytochemical techniques can support the conjectural diagnosis of 'preleukemia'.

## *Materials and Methods*

From 1968 to 1976 we examined blood and bone marrow smears of 63 patients suffering from partial or complex bone marrow failure using the routine cytochemical techniques listed below. Now we revised these cases, in 40 of which the diagnosis of 'probable preleukemia' had been made on the basis of preliminary clinical informations and of the peculiar cytochemical and morphological anomalies of the blood and bone marrow cells. In further 2. cases, the abnormal cytochemical and morphological patterns were absent or faint and therefore no diagnosis of 'probable preleukemia' had been made.

*A further case, included into the 'non-preleukemia' group, needs more extensive presentation (Case F).* A 32 year-old woman was studied because of severe complex bone marrow failure. The bone marrow aspirate yielded scarce hemopoietic bone marrow. The cytochemical and cytological examinations disclosed much of the features regarded as significant criteria for 'preleukemia'. However this diagnosis was not made because we had been impressed by the presence in the bone marrow of conspicuous large macrophages – 'histiocytes' – showing strong phagocytosis of bone marrow cells and erythrocytes. The absence of hepatosplenomegaly and the rather benign course argued against the diagnosis of a 'histiocytic medullary reticulosis' [20, 39], and we concluded for an etiologically unexplained 'hemophagocytic myelosis'. However 8 months later typical AML developed. This case was not included into the 'probable preleukemia' group, although objectively marked maturation defects were present at the time of the first examination. The case will be described in detail elsewhere [38].

15 patients of the preleukemia group and 8 of the 'non-preleukemia' group could not be followed adequately or are still alive without modification of the original diagnosis (fig. 1).

*In addition to these cases, we checked all patients admitted with AML to our clinic for a previous examination of their bone marrow function. It resulted that* none of these patients had been examined at the medical department during the 5 years preceding the manifestation of AML.

Smears of two further patients have been submitted from the First Department of Medicine, University of Munich, and the Department of Pediatrics, University of Innsbruck.[1] In both cases, we correctly made the diagnosis of 'preleukemia'. The latency periods were more than 4 months. The cytochemical and cytological anomalies were essentially the same as in the cases listed in table I and as discussed be

[1] We are indebted to PD Dr. H. HUHN, University of Munich, Department of Hematology, and to Doz. Dr. R. KURZ, Department of Pediatrics, University of Innsbruck.

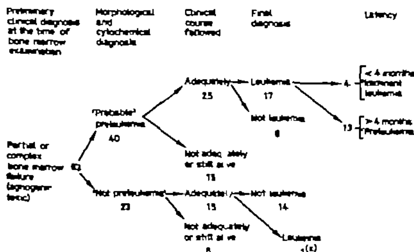



*Fig. 1.* Cases studied for the present investigations (May 1968 to May 1976). (X) = 'Hemophagocytic myelosis' see case report (case F.).

low. However, these patients have not been included into table V and into the other evaluations of our own patients.

None of the cases included in the 'probable preleukemia' group showed accumulation of blasts or of another uniform leukemic cell population in the bone marrow and no myeloblasts or atypical promyelocytes could be detected in the peripheral blood. According to our opinion, blast counts in the bone marrow exceeding 8% of the nucleated cells and presence of myeloblasts in the peripheral blood justify the diagnosis 'smouldering leukemia'. The clinical findings of the patients included to the 'imminent leukemia' group (fig. 1) closely corresponded to those of the 'true preleukemia' group summarized in table I and those of the 'aplastic anemia' group listed in table II.

The following staining procedures have been used: May-Grünwald-Giemsa, peroxidase, using as substrates 3-amino-9-ethyl-carbazole [33] or benzidine [14]; sudan black B [41]; naphthol-AS-D-chloroacetate esterase [23] as test for neutral protease activity [32]; acid phosphatase [1]; alkaline phosphatase [13]; sodium fluoride-sensitive and -resistant naphthol-AS-D acetate esterases [35]; α-naphthol-acetate esterase [35]; PAS reaction [21]; Perls' reaction for iron [3]. The cytochemical tests have been carried out after appropriate pretreatment of the smears. The nuclei have been counterstained either with acid Mayer's hemalum or with the Feulgen reaction.

## *Results*

(1) The cytochemical defects of the primary granules of the neutrophils include (a) quantitative differences of peculiar enzyme activities

*Table I* Clinical and laboratory findings in 'true preleukemia'

| Patients | Age, years | Sex | Bone marrow cells | Hemoglobin, g/dl | Hematocrit, vol % | Erythrocytes 10 /µl | Reticulocytes, /‰ | Thrombocytes × 10 /µl | Leukocytes 10³/µl | neutrophils/µl PMN + band-formed | neutrophils/µl Myelocytes + metamyelocyt. | Eosinophils/µl | Basophils/µl | Monocytes/µl | Lymphocytes/µl | Plasma cells/µl | Hepatomegaly | Splenomegaly | Lymph nodes | Final or autoptic diagnosis[1] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SK | 67 | m | n | 8.6 | 28 | | 22 | 69 | 2.0 | 1 030 | 120 | | | 50 | 800 | | + | | | |
| SN | 64 | m | ↓ | 7 4 | 24 | | 4 | 234 | 4.5 | 3 173 | | 45 | | 45 | 1,237 | | | | | |
| TJ | 53 | m | ↓ | 5 1 | 20 | | 6 | 25 | 2.1 | 231 | 21 | 21 | | 21 | 1 806 | | | | | |
| GE | 20 | f | ↓ | 12.3 | 34 | 3 1 | 3 | 15 | 4 1 | 1 661 | 123 | | 20 | 123 | 2,173 | | | | | AML |
| UJ | 66 | m | ↓ | 10.4 | 36 | 3 8 | 2 | 81 | 1 8 | 612 | | | | 36 | 1 152 | | | | | |
| RJ | 35 | m | ↑ | 6.1 | | 2.1 | 6 | 15 | 1 1 | 429 | 165 | | | | 506 | | | | | |
| AJ | 60 | m | ↓ | 5.2 | 17 | | 1 | 161 | 8 1 | 5,994 | 162 | 81 | | 162 | 1 701 | | | | | |
| WK | 40 | m | n | 6.5 | | 1 9 | 8 | 37 | 1.3 | 611 | 39 | 13 | | 104 | 533 | | | | | |
| NF | 38 | m | ↓ | 7 8 | 23 | 1 9 | 22 | 10 | 2.2 | 1 166 | | | 33 | 99 | 880 | | | | | smouldering AML |
| BR | 66 | m | ↓ | 7 7 | 24 | 2.5 | 37 | 6 | 3 1 | 837 | | 31 | | 434 | 1 798 | | | | | |
| PH | 67 | m | ↑ | 5.5 | 18 | | 2 | 22 | 8 4 | 5 460 | 252 | 84 | 168 | 840 | 1,596 | | + | + | | AMoL |
| WJ | 61 | m | ↓ | 11 0 | 34 | | 11 | 18 | 4 8 | 2,400 | 168 | | | 864 | 1,368 | | | | | SMML |
| MW | 66 | m | ↓ | 11.2 | 35 | | 16 | 42 | 4.2 | 2,184 | | 21 | 21 | 168 | 1 785 | | + | | | SMML |

[1] n = Normal ↑ = increased ↓ = decreased.
AMoL = Acute monocytic leukemia SMML = subacute myelomonocytic leukemia.

*Table II* Clinical and laboratory findings in aplastic anemia (= non-preleukemia group)

| Patients | Age, years | Sex | Bone marrow cells | Hemoglobin, g/dl | Hematocrit, % | Erythrocytes, $10^6/\mu l$ | Reticulocytes, ‰ | Thrombocytes, $10^3/\mu l$ | Leukocytes, $10^3/\mu l$ | neutrophils, /µl: PMN band-formed | neutrophils, /µl: Myelocytes + metamyelocytes | Eosinophils/µl | Basophils/µl | Monocytes/µl | Lymphocytes/µl | Plasma cells/µl | Hepatomegaly | Splenomegaly | Lymph nodes | Final or autoptic diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VC | 27 | m | ↓ | 9.9 | 30 | | 1 | 1.0 | 0.3 | | | | | | 300 | | | (+) | | acute aplastic anemia |
| LM | 17 | f | ↓ | 7.0 | — | | 0 | 13.0 | 4.0 | 1,880 | 140 | 40 | | 80 | 1,840 | 20 | | | | |
| SM | 68 | f | | 9.0 | 30 | | 0.5 | 234.0 | 2.6 | 780 | | 390 | 39 | 208 | 1,183 | | | | | |
| SJ | 58 | m | ↓ | 9.2 | | 3.3 | 4 | 6.0 | 2.4 | 288 | | | | 96 | 2,016 | | | | | |
| KJ | 64 | m | | 6.9 | 22 | | 7 | 7.0 | 1 | 861 | | 32 | | 126 | 1,081 | | | | | |
| PM | 54 | f | ↓ | 7.6 | 24 | 2.3 | 17 | 3.0 | 2.1 | 232 | | 63 | | 126 | 1,659 | | | | | |
| RF | 67 | m | | 7.0 | 19 | | 31 | 37.0 | 1.0 | 630 | 10 | | 10 | 10 | 345 | 5 | | | | |
| SA | 64 | m | ↓ | 10.0 | 32 | 3.4 | 8 | 11.0 | 0 | 1,030 | 20 | 20 | | 20 | 910 | | | | | |
| FK | 15 | m | ↓ | 10.1 | 30 | 2.8 | 34 | 16.0 | 5.4 | 1,296 | 54 | 54 | | 540 | 3,456 | | | | | |
| KA | 53 | f | | 11.9 | 38 | | 4 | 9.0 | 1.4 | 856 | 8 | 88 | | 16 | 444 | 8 | | | | drug-induced? aplastic anemia |
| SF | 62 | m | ↓ | 7.5 | 24 | 2.0 | 4 | 7.0 | 1.3 | 514 | | | | | 767 | 19 | | | | |
| HO | 45 | f | ↓ | 5.9 | 17 | | 2 | 9.0 | 1.4 | 336 | | | | 14 | 1,050 | | (+) | | | |
| ZC | 77 | f | ↓ | 9.4 | 28 | | 30 | 13.0 | 3.0 | 1,560 | 60 | 30 | | 30 | 1,320 | | | | | Immunoblastic lymphoma and aplastic anemia |
| RR | 31 | m | ↓ | 7.5 | 26 | 2.6 | 14 | 15.0 | 1.1 | 506 | 11 | | | 33 | 550 | | | | | |

= Normal ↓ = decreased.

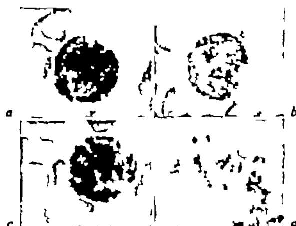



Fig. 2. Naphthol AS-D-chloroacetate esterase activity in the PMN neutrophils of normal subjects (a) decreased activity may occur in severe bacterial infections (b) in preleukemia the PMN neutrophils may show moderately or markedly decreased enzyme activity arranged in coarse granules or in clusters (c, d). Orig. magnif × 1,200.

(peroxidase and neutral protease) among the polymorphonuclear neutrophils (PMN N) of the same patients very weakly stained or unstained PMN N have been termed paraneutrophils [36] (b) coarse granules or clustered cytochemical reaction products, which are due to the altered cytoplasmic distribution of constituents and precursors of the primary granules [36]

The best evidences for these anomalies have been obtained using the tests for neutral protease activity employing the substrate naphthol AS-D-chloroacetate (fig 2) Closely corresponding results are usually obtained following the staining for peroxidase or with sudan black B Localization of peroxidase activity was even better using the latter staining procedure than employing 3 amino-9-ethyl-carbazole as the substrate for peroxidase activity

(2) A further remarkable anomaly was the occurrence in some cases of unexpectedly low indices of the leukocyte alkaline phosphatase (LAP) activity [7 18 34]

(3) Glycogen – as shown using the PAS reaction – appears evenly distributed in the normal PMN N A granular distribution can occasionally be observed in leukemia, in chronic infections and in preleukemia (fig. 3)

(4) In monocytes the atypical traits possibly due to the preleukemi perturbation of the maturation include the presence of fine or coarse gran

*Table III.* Some primary and secondary hematologic disorders showing cytochemical and cytological evidence for disturbed maturation

| Faint anomalies | Intermediate anomalies | Marked anomalies |
|---|---|---|
| Chronic inflammatory diseases (bacterial infections, rheumatoid disorders, some viral infections) | Viral infections | Leukemia |
| | Treatment with occasionally myelotoxic drugs | Preleukemia |
| | Polycythemia vera | PNH |
| | Splenomegalic bone marrow depression | Myeloid metaplasia |
| | | Congenital disorders of the myelopoiesis |
| | | Mastocytoses |
| | | Treatment with cytotoxic drugs |
| | | Treatment with diphenylhydantoin |
| | | Hodgkin disease |
| | | Tuberculosis |
| | | Alcoholism |
| | | Chronic aggressive hepatitis |
| | | Malabsorption |

ular deposits of glycogen as well as low and irregularly distributed naphthol-AS-D-acetate esterase. The latter finding is especially notable if considerable quantitative dissociation of the usually similar activities of nonspecific esterase and acid phosphatase can be recognized.

(5) Increased numbers of weakly or strongly stained sideroblasts is one of the most frequently reported anomalies in preleukemia [7, 18, 23, 31, 34, 46, 48]. The degree of the sideroblastosis may vary within a wide range. Presence of granular PAS-positive materials in immature erythroblasts and proerythroblasts is a further common finding [7, 34] (fig. 4). Mature erythroblasts occasionally show a diffuse staining [22]. Marked to strong perinuclear α-naphthol-acetate esterase and strong paranuclear staining for acid phosphatase usually occur in the megaloblasts and macroblasts of erythremia and erythroleukemia, but a similar profile may also be present in preleukemia.

None of the cited atypical traits *per se* directly implies preleukemia. Similar anomalies, although usually less distinct and striking, can be observed in definitely non-leukemic conditions such as those listed in table III. Based on the preliminary clinical diagnosis of 'partial' or 'complex bone marrow insufficiency', we concluded that in 40 patients the observed abnormal cytochemical patterns of the hemopoietic cells argued




Fig. 2. Naphthol AS-D-chloroacetate esterase activity in the PMN neutrophils of normal subjects (a) decreased activity may occur in severe bacterial infections (b) In preleukemia the PMN neutrophils may show moderately or markedly decreased enzyme activity arranged in coarse granules or in clusters (c, d). Orig magnif × 1,200.

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*Table IV* Comparison of the cytochemical findings observed in the blood and bone marrow cells in preleukemia and in aplastic anemia (= 'non-preleukemia')

| Results | Preleukemia | Aplastic anemia |
|---|---|---|
| Index of the LAP | | |
| High | 10 | 13 |
| Normal | 2 | 1 |
| Low | 3 | 0 |
| Atypical primary granules | 13/15 | (2) 14 |
| Atypical PAS positivity | | |
| Granulocytes | 3/15 | 0/14 |
| Erythroblasts | 5/15 | 0/14 |
| Stem cells | 4/15 | 0/14 |
| Sideroblastosis | 6/15 | 0/14 |
| Pathologic erythroblastic hydrolases | 3/15 | 0/14 |
| Atypical monocytes | 6/15 | 0/14 |

for the presence of a preleukemic syndrome ('probable preleukemia') Out of the 25 adequately followed patients (fig 1), 4 times leukemia developed within 4 months ('imminent leukemia') In 13 cases, acute leukemia (AL) or subacute myelomonocytic leukemia (SMML) or smouldering leukemia (SL) manifested 4 or more months after the diagnosis ('preleukemia'). No evident differences of the cytochemical anomalies occurred among the cases included in the imminent leukemia and the 'preleukemia groups.

We compared the cytochemical patterns of the hemopoietic cells in 15 cases of preleukemia with those of 14 cases of aplastic anemia. The differences among the two groups are shown in table IV In each case of 'preleukemia one or more identifying criteria occurred in varying combinations (table V) These peculiar features were absent and the alkaline phosphatase activity was almost always high if the patients later died without the manifestation of leukemia.

### *False Positive Diagnosis of Preleukemia*

We would like to emphasize again that the diagnosis 'probable preleukemia was based on preliminary clinical informations, such as 'anemia 'thrombopenia or 'neutropenia 'bone marrow insufficiency and on the

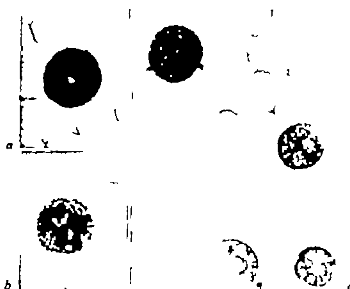


*Fig 3* PAS stain *a* Normal PMN neutrophil *b* PMN neutrophil showing coarse granular distribution of PAS-positive materials. *c* PMN neutrophils with coarse granules of PAS-positive materials in myeloblastic leukemia. Orig. magnif × 1,200.

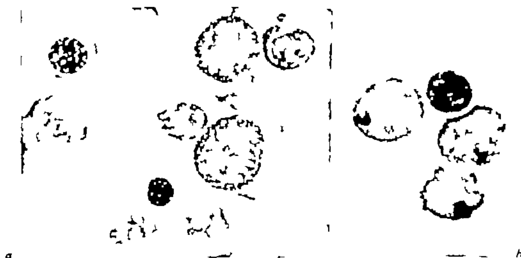


*Fig 4* Abnormal cytochemical profiles in erythroblasts observed in preleukemia. *a* Granular PAS positivity in early erythroblasts. *b* Paranuclear spot of strong acid phosphatase activity Orig. magnif. × 1,200.

*Table IV* Comparison of the cytochemical findings observed in the blood and bone marrow cells in preleukemia and in aplastic anemia (= 'non-preleukemia')

| Results | Preleukemia | Aplastic anemia |
|---|---|---|
| Index of the LAP | | |
| High | 10 | 13 |
| Normal | 2 | 1 |
| Low | 3 | 0 |
| Atypical primary granules | 13/15 | (2)/14 |
| Atypical PAS positivity | | |
| Granulocytes | 3/15 | 0/14 |
| Erythroblasts | 5/15 | 0/14 |
| Stem cells | 4/15 | 0/14 |
| Sideroblastosis | 6/15 | 0/14 |
| Pathologic erythroblastic hydrolases | 3/15 | 0/14 |
| Atypical monocytes | 6/15 | 0/14 |

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*False Positive Diagnosis of Preleukemia*

We would like to emphasize again that the diagnosis 'probable preleukemia was based on preliminary clinical informations, such as 'anemia thrombopenia or 'neutropenia 'bone marrow insufficiency and on the

*Table I.* Occurrence of the various cytochemical anomalies among the patients being in a preleukemic stage

| | | PMN neutrophils | | Erythroblasts | | | | PMN neutrophils | | | Erythroblasts | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bone marrow cellularity[1] | atypical granules | cytoplasmic anomalies | megalo-macroblastosis | nuclear anomalies | Monocytosis | Micromegakaryocytes | atyp. primary granules | LAP index[1] | gran. PAS positivity | PAS positivity | sideroblastosis | hydrolase anomalies | Atypical monocytes |
| SK | n | • | | | | | | • | n | • | | | | |
| SN | ↓ | | | | | | | | ↑ | • | | • | | |
| TJ | ↓ | | | | | | | • | ↑ | | • | | | • |
| GE | ↓ | | • | | • | | | | ↓ | | • | | | |
| UJ | ↓ | | | | | | | • | ↓ | | | | | |
| RJ | ↑ | | | | | | | • | ↑ | | | | | |
| AJ | ↓ | | | | | | • | • | ↓ | | | | | • |
| WK | n | | | • | • | | | • | ↑ | | • | • | • | |
| NF | ↓ | | | • | | | • | • | ↑ | | | • | | |
| BR | ↓ | | • | | | | | • | ↑ | | | | • | |
| PH | ↑ | • | | • | | • | • | • | ↑ | | | | | • |
| WJ | ↓ | | | • | | • | | • | ↑ | | | • | | • |
| MW | ↓ | | | | | | | • | ↑ | | • | | | • |

n = Normal ↑ = increased ↓ = decreased

cytological and cytochemical findings Among the 25 adequately followed cases of 'probable preleukemia in 8 the diagnosis was erroneus. The final and/or autoptic diagnosis of these patients include tuberculosis (2 cases) treatment with diphenylhydantoin (1 case) paroxysmal nocturnal hemoglobinuria (1 case) alcoholism and moderate liver damage (1 case) chronic aggressive hepatitis (1 case) Hodgkin s disease (2 cases)

*Erroneously Negative Diagnosis of Preleukemia*

Besides one exceptional case which we described in detail in Materials and Methods no false negative diagnosis occurred in our materials. In a further case the exact diagnosis was delayed the 64-year-old patient (ta

ble I case UJ) was thought to suffer from aplastic anemia for more than 1 year The abrupt decrease of the consistently high LAP scores to subnormal levels led us to suspect a preleukemic syndrome despite the absence of any further cytological or clinical changes. During the following months a few further cytochemical anomalies of the PMN-N in the peripheral blood became manifest. True leukemia developed 8 months after the first diagnosis of 'preleukemia

## *Discussion*

*Cytochemically Detectable Anomalies in 'Preleukemia'*

Several authors reported on the occurrence of abnormal cytochemical patterns in blood and bone marrow cells of patients in which years or several months later AL developed [7 10 17 18 29–31 34 46] Disorders of the RNA metabolism are the most likely causes of these cytochemical anomalies, which are related to the disturbed maturation capacity of the affected cell clones. Striking disorders of the RNA metabolism have been detected in AL [12, 28, 44] Although such detailed investigations have not been undertaken in preleukemia, it is conceivable that essentially the same factors may be active in producing the faulty cytologic maturation. Evidence exists that in preleukemia the cellular proliferation is also disturbed [11] The reported anomalies in preleukemia can be classified according to the following groups:

(a) defective enzyme equipments of apparently mature cells, these include decreased cytochemically demonstrable activities of peroxidase, chloracylesterase, alkaline phosphatase in PMN-N decrease of nonspecific esterase and acid phosphatase in monocytes impaired hemoglobin production in erythroblasts

(b) accumulation of various materials due to defective synthetic activity of the preleukemia cells, i.e., iron deposits in sideroblasts the accumulation of polysaccharides in erythroblasts and in some other hardly classifiable blasts may also belong to the same kind of disorders,

(c) unusual increases of cytoplasmic enzyme activities, such as alkaline phosphatase in PMN N and acid phosphatase and nonspecific esterase in erythroblasts,

(d) defective cytoplasmic organization this may be shown in some PMN-N showing coarse granular distribution of PAS-positive materials and atypical distribution of enzymes peculiar for the primary granules

(e) quantitative cytochemistry revealed considerable anomalies of the DNA content in megakaryocytes [29]

*Can the Suspect Diagnosis of Preleukemia be Supported by the Cytochemical Examination of Blood and Bone Marrow Cells?*

It is worth emphasizing that the cytochemical examination of bone marrow smears generally greatly improves the diagnostic value of bone marrow aspirations This refers both to the now widely accepted cytochemical classification of the acute leukemias as well as to the cytochemical evaluation of the bone marrow cells in primary and secondary disorders of the myelopoiesis. The cytochemical tests are usually distinctly more sensitive in detection of maturation defects than the conventional cytological examination. The evidentiation of single enzyme activities or of accumulated organic or anorganic materials allows the more subtile evalution of the abnormal cell features. The detection of abnormal cytochemical profiles and the degree of the detected anomalies may argue for the presence of a preleukemic syndrome which may be suggested by the clinical course and by the morphologic findings.

Based on the cytological and cytochemical findings and on a few preliminary clinical data we concluded for the diagnosis of preleukemia in 40 cases. Out of the 25 adequately followed cases AML, SMML or SL developed in 17 in 8 cases the observed maturation defects could not be attributed to leukemia

*False Positive Diagnosis of Preleukemia*

Cytochemically detectable anomalies in the PMN N may be congenital [27 36 45] or may occur in some bacterial and viral infections. Further maturation defects of the myeloid cells can be caused by several drugs. However with a few exceptions – for instance, those due to diphenylhydantoin, which is thought to interfere with the folic acid metabolism – these anomalies are usually not as marked and they usually are not as suggestive for preleukemia Following the treatment with cytotoxic drugs excessive anomalies of the hematopoiesis have been observed, which in some cases were even more striking than those observed in preleukemia Further primary and secondary hematologic disorders which may be associated with maturation defects have been listed in table III

The anomalies observed in tuberculosis and in Hodgkin s disease are difficult to explain Both hepatic dysfunction and the viral infection may concur in producing the bone marrow depression frequently seen in

chronic aggressive hepatitis. The toxic effect of alcohol on the hematopoiesis may be mediated by the hepatic dysfunction, although a direct toxic effect has been discussed [37] Studies concerned with the frequency the type and the degree of the anomalies in alcoholism, tuberculosis, and Hodgkin s disease are in progress The myeloid defects in paroxysmal nocturnal hemoglobinuria (PNH) and in myeloid metaplasia are probably intimately related to those in leukemia. The development of leukemia as the final event in these as well as in some cases of myelofibrosis is well known.

*False Negati e Diagnosis*

We reported on a woman in whom, despite the presence of prominent cytochemical anomalies, we had been impressed by the strong phagocytosis of bone marrow cells and therefore had made the diagnosis of hemophagocytic myelosis [20] However AL developed in this patient after 8 months. In a further patient, the diagnosis agnogenic aplastic anemia had been changed to preleukemia 8 months before manifestation of leukemia. If we disregard the first case – which objectively showed strong features suggesting preleukemia as described above – it can be concluded that no false negative diagnosis had been based on the cytochemical examinations. It emerges from our results that, if appropriate examinations are performed, actually in all cases of AML cytochemical and cytological anomalies could be detected several months before the manifestation of the true disease. Furthermore, in some cases repeated examinations disclosed a progressive manifestation of anomalies. This suggests that the accumulation of myeloid leukemic cell populations is preceded by a progressive maturation disorder of myeloid cell clones.

*Diagnosis of Preleukemia*

This diagnosis should be based on the following criteria (1) presence of single or combined disorders of the myeloid cell lines, (2) careful anamnestic and clinical evaluation and exclusion of the myelopoietic disorders discussed in the previous chapter (table III) (3) absence of a relevant accumulation of blasts (less than 3 / of the nucleated bone marrow cells) or of other monomorphic cell populations (4) presence of marked cytochemical and cytologic anomalies in several myeloid cell lines (table V) The meticulous cytochemical and cytological examination may allow to distinguish between preleukemia and related atypical myeloproliferative diseases and not leukemia-associated bone marrow disorders (table

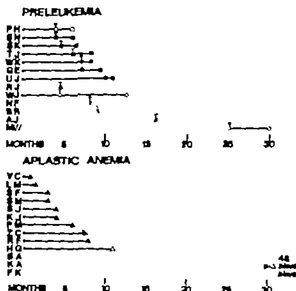



*Fig 5* Survival in preleukemia and in aplastic anemia. ■ = AML, ● = smouldering myeloblastic leukemia □ = acute monocytic leukemia O = SMML, ▽ = manifestation of leukemia ▲ = agnogenic aplastic anemia, △ = drug associated aplastic anemia ⅃ = lymphoma-associated aplastic anemia.

III) if only single cytochemical tests are employed, the diagnostic useful ness is rather poor [18] (5) nevertheless, in a few cases it may be impossible to distinguish between preleukemia and mimicking disorders. Initial monthly controls and simultaneous treatment of associated disorders may further clarify the myelopoietic defect. The diagnosis 'preleukemia cannot exclude that in other areas of hematopoiesis not studied rudimentary accumulation of a leukemic cell clone already occurred

*Clinical Value of the Diagnosis Preleukemia*

According to the published data [4 18 19] and to our own experience the duration of the preleukemic stages may largely vary and may extend even to several years However as leukemia has become manifest, the course is usually rapidly fatal and induced remissions occur very exceptionally

Only a few out of the cases of agnogenic or secondary bone marrow insufficiency had a favorable course (table II fig. 5) By contrast, most other cases of aplastic anemia showed a brief survival If the first 10 cases of aplastic anemia in figure 5 are taken as a group, the mean survival is considerably shorter (4 7 months) than in preleukemia (11 8 months)

Strikingly preleukemia has been diagnosed much more frequently in men than in women [19] In our materials – if only the cases of true pre leukemia are regarded – *the relation is 12 men to 1 woman, or – if the* cases of 'imminent leukemia' are added – 14 3 At the present state of knowledge on the etiology and the pathogenesis as well as on the treat ment of leukemia, the value of the diagnosis of preleukemia is still a mat ter of debate *In our opinion, preleukemia should not be treated with cy*totoxic drugs, or if the case, should be treated very carefully Instead, immunotherapy seems to us to be an approach which deserves discussion. A further possible treatment may consist in the transplantation of bone mar row Controlled trials on comparably diagnosed preleukemia cases are needed for answering these problems. Very valuable diagnostic supports may be given by cytogenetic examinations and especially by agar cultivation of bone marrow cells [16 24 25 40 43] Liquid cultures also yield ed promising results [5 6]

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# Morphological Differences in Human Peripheral Blood Lymphocytes

## A Freeze-Etching and Scanning Electron Microscopy Study

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*Key Words.* Lymphocytes · Freeze-etching · Scanning electron microscopy · Plasma membrane

*Abstract.* A morphological study using freeze-etching and scanning electron microscopy was carried out on lymphocytes from human peripheral blood.

Freeze-etching electron microscopy of lymphocytes fractured at the level of the membrane shows the presence of two groups of cells according to their surface morphology: lymphocytes with a smooth surface and lymphocytes with a villous surface. Cells with surface features identical to the two types described by freeze-etching were also observed by scanning electron microscopy. Some lymphocytes with smooth surface showing uropods were also observed.

## *Introduction*

Some workers using scanning electron microscopy (SEM) have reported that B lymphocytes have villous surfaces and that T lymphocytes are largely smooth [6, 7, 12–14]. More recently, studies with SEM claimed that villous surfaces are found in both lymphocyte populations [1, 2]. This discrepancy has been attributed to artefacts derived from the method of cell collection as well as to the different preparative techniques used in SEM [1, 2]. No physical fixation methods have been used to clarify this problem. It seemed, therefore, proper and in order to contribute to a better understanding of the surface morphology of human peripheral blood lymphocytes (PBL) to use freeze-etching, a technique specially suited to

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study cell membranes. The results obtained in these studies, performed with PBL, are summarized in the present report and compared with SEM observation of the same cells.

## Methods

*Lymphocytes*

40 ml of peripheral blood were drawn from a healthy young donor and lymphocytes were isolated from the heparinized blood by plasma filtration on nylon columns. Differential counts showed 95% lymphocytes and 5% monocytes. Three samples of blood from the same donor were studied by freeze-etching and SEM

*Cell Collection*

For freeze-etching the lymphocyte suspensions were centrifuged at 200 g for 10 min, and the cell pellet was resuspended in 0 1 M cacodylate-Cl buffer pH 7 4 with 30% glycerine. After 30 min at room temperature, the suspensions were centrifuged at 200 g for 15 min and portions of the cell pellet mounted on gold discs.

For SEM the lymphocyte suspension was immediately allowed to settle in Leighton tubes containing monolayers of BHK cells grown over glass slides [8] After 60 min at 37 C (to allow adhesion of lymphocytes to BHK cell surfaces) the slides were removed and fixed for SEM

*Cell Fixation and Observation*

After immersion of the gold discs, first in Freon 22 (– 155 C) for 5 sec and then in liquid nitrogen (– 196.3 C), conventional freeze-etching was carried out using a Balzers BA 360 M apparatus. The etching time period was 1 min at – 100 °C. Replicas were observed in a Philips EM 300 electron microscope at 40 kV

For SEM the slides were fixed with 2% glutaraldehyde in 0.1 M cacodylate-Cl buffer pH 7 4 rinsed in the same buffer postfixed with 1 % osmium tetroxyde in veronal acetate buffer pH 7 4 Sucrose was added to the last solutions to obtain the same osmolarity as the other solutions used earlier (410 mosm). Dehydration was carried out in a graded series of ethanol followed by an acetone wash and critical point drying using CO The slides were coated with carbon and gold in a vacuum unit and observed at an angle of 45 in a Jeol 50 A SEM at 25 kV

## Results and Discussion

Freeze-etching electron microscopy of PBL fractured at the level of the membrane clearly shows according to their morphology the presence of cells with a villous surface (fig 1a) and of cells with a smooth surface (fig. 1c 2a) Faces of cell membrane showing intramembranous particles (IMP) were studied in order to determine the density of these particles no

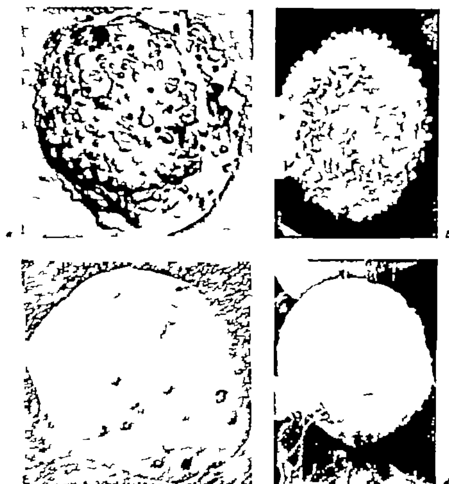


*Fig. 1.* *a.* Villous lymphocyte visualized by freeze-etching. Its surface is covered by multiple microvilli. × 7,000. *b.* SEM micrograph of villous lymphocyte. 6,400. c. Freeze-etching micrograph of smooth lymphocyte. 6,600. *d.* Smooth lymphocyte. SEM × 6,000.

differences between the two groups of cells were found (table I). Lymphocytes with surface features identical to those described above were also observed by SEM (fig. 1b, d). Moreover the results show that 70% of human PBL had a smooth surface whereas 20% were villous (table I). Some cells with smooth surfaces showing uropods were also observed (fig. 2b).

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*Table I* Morphological characteristics of human peripheral blood lymphocytes as seen by SEM and freeze-etching (mean ± SD)

| Characteristics | Smooth cells | Villous cells |
|---|---|---|
| *SEM*[1] | | |
| Percent of cells | 70 | 20 |
| Cell size, µm | 5.18 ± 0.56 | 5.07 ± 0.47 |
| Microvilli size, µm | | 0.20 ± 0.02 |
| Microvilli length, µm | | 0.99 ± 0.12 |
| of microvilli per exposed surface | | 112 ± 12.3 |
| *Freeze-etching* | | |
| IMP diameter Å | 82.9 ± 8.45 | 84.3 ± 13.1 |
| IMP density IMP µm | 280 ± 32.4 | 295 ± 34.7 |
| IMP distribution | monogranular | monogranular |

200 cells from each sample

Student t test was used for statistical comparison of the IMP density ( of villous cells = 35 of smooth cells = 27 $p < 0.05$).

[1] According to MANDEL [9]

has been made by both light and transmission microscopy [10 15 16] Obviously and given the importance of the subject, further work should be carried out.

## Acknowledgements

I am grat ful to Dr J HERNANDEZ, Dr A MARTINEZ, Dr M. BLANCO, Dr E. KNIGHT and particularly to Dr A POLLIACK, Dr C. PASTERNAK and Dr S. GENOLLA for constructive criticism during the preparation of this manuscript.

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3 CHIPWELL, B S RICH, R. R DARDANO, J and KIMZEY S. L Scanning electron

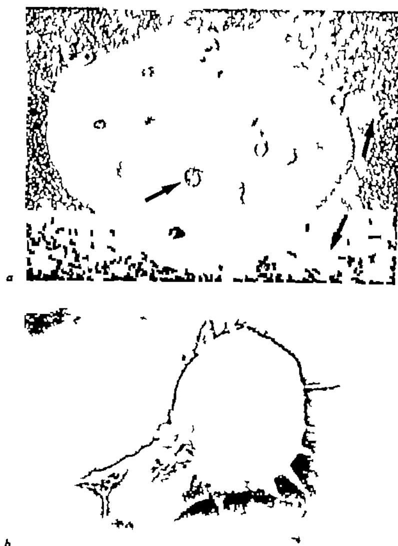



*Fig 2* a. Smooth lymphocyte visualized by freeze-etching showing some fractured microvilli (arrows) × 6,800. *b* Uropod forming cell. The surface of the cellular body is smooth. × 5,000

In view of these observations and although modifications of the cell surface occur in some cells, including lymphocytes, during the cell cycle and during preparation techniques [3 4 5 8 11] these do not explain the observations presented here and the excellent correlation in the images obtained by both techniques, using completely different methods of cell collection and fixation Moreover a similar division of human PBL

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| IMP density IMP µm | 280 ± 32.4 | 295 ± 34 7 |
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## *Acknowledgements*

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Acta haemat. 59: 25–30 (1978)

# Unusual Lymphocyte Morphology in a Case of Chronic Lymphatic Leukaemia: Apparent Nuclear Extrusion

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*Key Words.* Lymphocyte morphology · Scanning electron microscopy · Transmission electron microscopy · Chronic lymphatic leukaemia

*Abstract.* The unusual lymphocytes from a case of chronic lymphatic leukaemia are described in terms of surface morphology and ultrastructure. Surface blebs were found to consist of nuclear material surrounded by plasma membrane. On the information available it was not possible to determine whether these were due to cell motility or nuclear extrusion.

Peripheral blood from lymphocytes from patients with chronic lymphatic leukaemia (CLL) are examined in this department using the scanning and transmission electron microscopes. The recent development of improved techniques which have reduced the possibility of preparative artefacts [NEWELL *et al.*, 1976] have allowed us to make detailed observations on the surface and ultrastructural morphology of these cells. The peripheral blood lymphocytes from one particular patient illustrated a phenomenon unique in our experience; the structural studies on these cells are described and discussed.

## *Materials and Methods*

Peripheral blood lymphocytes were isolated by Ficol/Triosil gradient centrifugation and prepared for electron microscopy as previously described by NEWELL *et al.*

*We would like to thank Dr. R. Hyde and Dr. J. Bamforth for referring this patient for morphological studies.*

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[1976] The cell suspension was processed for scanning electron microscopy (SEM) using an adapted BEEM capsule [NEWELL and ROATH, 1975] and also embedded in Spurr resin and sectioned for transmission electron microscopy (TEM).

Surface marker studies were performed on lymphocyte suspensions for the identification of cells with receptors for Fc and sheep erythrocytes by rosette tests and surface immunoglobulin by immuno-fluorescent staining. These methods are described elsewhere [SMITH and HAEGART 1974].

## *Clinical History*

A 70-year-old retired brewer's drayman with a history of modest alcohol consumption was seen by his family doctor having had diarrhoea and abdominal pain for weeks. Gross splenomegaly and hepatomegaly were found and the patient was referred for investigation. The diarrhoea, which was thought to have been infective in origin, ceased spontaneously.

Blood haemoglobin was 1 g/dl total white blood count was $4.0 \times 10^9$/l with $2.8 \times 10^9$/l small lymphocytes and peatelets were $93 \times 10$ /l. Sternal marrow obtained showed 40% of the cells to be small lymphocytes. Liver biopsy was compatible with CLL but also revealed the presence of some strongly PAS-positive rounded bodies, 5 μm in diameter within some parenchymal cells. This was thought compatible with $\alpha$ anti-trypsin deficiency which was confirmed by a blood level of 100 U (normal 180–400 U). Lung function tests were normal and liver function tests showed normal aspartate transaminase, lactic dehydrogenase and bilirubin, but raised alkaline phosphatase (46 KAU) and 5-nucleotidase of 62 IU/l, which was unhelpful in excluding bone disease. A diagnosis of CLL in a man with partial $\alpha_1$ anti trypsin deficiency was made. His disease was stable over 3 years of observation but he developed iron deficiency during this period, confirmed after bone marrow examination. The partial anti-trypsin deficiency was apparently asymptomatic.

2 years after his original presentation he developed colicky abdominal pain which led to radiological investigation of the large bowel revealing a caecal tumour. Laparotomy confirmed the presence of a caecal neoplasm which was shown by histology to be an adenocarcinoma.

The patient had a stormy post-operative course with multiple infections and he died 10 days following operation with renal failure and acidosis. The observations reported here on his peripheral blood lymphocytes were made during this 10-day period.

## *Results*

### *Electron Microscopy*

By SEM a heterogenous population of lymphoid-like cells were observed with poorly developed microvilli on the cell surface. In addition almost every cell was seen to have one or two smooth bulbous projections between 1 and 4 μm in diameter (fig. 1). All these projections were seen

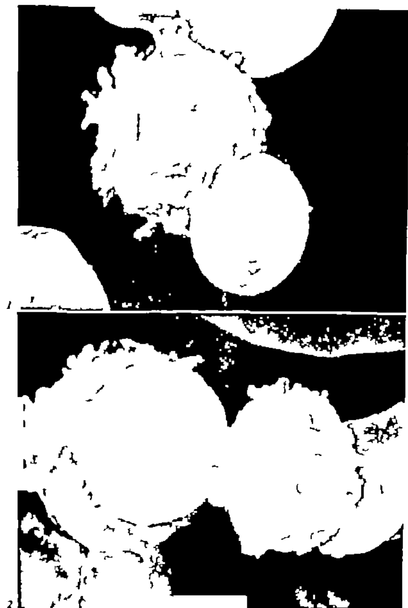



*Fig. 1* SEM of peripheral blood lymphocyte showing single bleb on the cell surface × 10,000

*Fig. 2* SEM showing the continuation of the plasma membrane over the surface bleb. × 10,000



4

*Fig 3* TEM of peripheral blood lymphocyte with a nuclear bleb × 11,800

*Fig 4* TEM showing the presence of many microtubules and microfilament around the nuclear material. × 11 800.

to be attached to the cell by *cytoplasmic extensions such that there was a* continuation of the plasma membrane (fig 2) Although the surface of each projection appeared smooth at low magnification, a roughened surface was observed at higher magnification as well as the occasional short stub-like microvillus.

Thin sections of the same cell preparation showed that these bulbous projections consisted almost entirely of nuclear material surrounded by a thin layer of cytoplasm and plasma membrane (fig. 3) The cytoplasm and cytoplasmic organelles in the nuclear clefts were retained in the projection. Microfilaments and microtubules were evident especially at the constriction between the nuclear material in the projections and that still remaining within the bulk of the cell (fig. 4) In both 1 μm and thin sections there were many small cells consisting almost entirely of nuclear material with little, if any cytoplasm, but it was not possible to establish conclusively that these were actually cytoplasm free membrane-bound nuclei or due to sectioning artefacts.

The patient's serum was found to be negative for antinuclear factor and did not induce the phenomena described when incubated with normal peripheral blood lymphocytes. Surface marker studies showed this patient to have a monoclonal proliferation of MDk surface immunoglobulin positive cells (70%) and 96% of the lymphocytes were found to have Fc receptors while only 3% were found to be T cells as detected by sheep red blood cell rosettes.

## *Discussion*

A case of CLL with unusual morphological features is described which involves the production of nuclear blebs enclosed by plasma membrane This phenomena may be explained by either lymphocyte motility resulting in the movement of the cytoplasm away from the nucleus or by the active expulsion of the nucleus from the cytoplasm.

Further studies on the lymphocytes of this patient could not be undertaken to allow the mechanism involved to be determined, and no other patient has shown more than an occasional instance of this phenomenon.

Lymphocyte motility has been described *in vitro* [Bessis, 1973] and *in vivo* in cells migrating through the lymphoid tissues [Van Ewijk *et al.*, 1975] Both processes involve the production of a mobile, front-like structure leaving the nuclear material at the rear of the cell. Such morphological distortions of cell shape are consistent with those observed by

TEM in this case However the scanning electron micrographs do not suggest the production of mobile organelles and are more suggestive of expulsion of nuclear material from the cell as they clearly show a sequence of events beginning with the extrusion of a small nuclear bleb and culminating in the separation of this bleb from the cytoplasm In this individual the phenomenon was seen in association with a tumour following surgery in an infected terminally ill patient Whether any or all of these associated states are concerned with the phenomenon and whether it only occurs in the lymphocytes of patients with CLL cannot be stated on the evidence we have presented If other observations of this type have been made elsewhere, collectively an explanation for the phenomenon might be found

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# Pure Red Cell Aplasia, Toxic Dermatitis and Lymphadenopathy in a Patient Taking Diphenylhydantoin

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*Key Words.* Pure red cell aplasia Lymphadenopathy Diphenylhydantoin

*Abstract* A patient taking diphenylhydantoin for 3 weeks developed a generalized skin rash, lymphadenopathy and pure red cell aplasia. After withdrawal of the pharmacon all symptoms disappeared spontaneously Skin rash is well-known complication of diphenylhydantoin treatment as is benign and malignant lymphadenopathy Pure red cell aplasia associated with diphenylhydantoin medication has been reported in 3 patients. The exact mechanism by which diphenylhydantoin exerts it toxic effects is not known. In this patient the time relation between the ingestion of diphenylhydantoin and the occurrence of the skin rash, lymphadenopathy and pure red cell aplasia is very suggestive of direct connection.

## *Introduction*

Diphenylhydantoin has been used for therapy in epilepsy since 1938 [12] Many side effects of the drug are known. The most common are gingival hyperplasia, dermatitis accompanied by fever and eosinophilia, hypertrichosis, gastrointestinal discomfort and cerebellar incoordination [3] Haematological complications like megaloblastic anaemia, leucopenia, pancytopenia, thrombocytopenia [19] and haemolytic anaemia [18] occur less often. Since 1959 many patients developing lymphadenopathy during diphenylhydantoin treatment have been described [4, 5 14 16, 17] there has arisen considerable doubt about the benignity of this lymphadenopathy [1 6, 8 10, 13] Until now 3 patients with pure red cell aplasia associated with diphenylhydantoin treatment have been reported [4 11] We report a patient who developed pure red cell aplasia during

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diphenylhydantoin therapy. Moreover she had transitory apparently benign lymphadenopathy and toxic dermatitis.

## *Case History*

A 16 year old Caucasian woman was admitted to our hospital (November 17 1973) because of fever, generalized skin rash and cervical and nuchal lymphadenopathy. She was well until November 18, when she experienced stiffness of the neck, fingers and feet. A week later she observed enlarged lymph nodes in the neck and a puffy face. Because of headaches capsules containing phenobarbital, diphenylhydantoin and coffein (60, 60 and 25 mg, respectively) three times daily were prescribed starting October 18. On November 6, a skin rash appeared and the body temperature rose to 38.5 °C. The capsules were withdrawn. During the next week body temperature rose to 40 °C and the colour of the skin rash turned to purple. The lymph nodes enlarged and became tender. She was referred to our hospital.

On physical examination the patient appeared ill, a purple, generalized skin rash was noted. Body temperature was 38.9 °C, blood pressure 135/70 and pulse rate 132/min. In the nuchal area enlarged, tender lymph nodes were palpated. The heart, lungs and breasts were normal. The liver was not enlarged, the spleen was felt just below the costal margin and tender. Neurologic examination was negative.

*Laboratory Findings*

On admission the ESR was 15 mm in the first hour, haemoglobin 9.– mmol/l, haematocrit 47%, reticulocyte count 0.6%, white cell count $13.2 \times 10^9$/l with 15% eosinophils, 1% band forms, 15% polymorphonuclears, 66% lymphocytes and 3% monocytes. The platelet count was normal. Serum levels of sodium, potassium, urea, creatinin and glucose were normal. Bilirubin was 81 µmol/l, alkaline phosphatase 335 U/l, SGOT 43 U/l, SGPT 96 U/l and LDH 595 U/l (upper limit of normal 9 µmol/l, 100 U/l, 15 U/l, 15 U/l and 175 U/l, respectively). Total protein, albumin and globulin were normal. A urinalysis was normal. Tests for antibodies against rubella virus, adenovirus, parainfluenza virus, influenza virus, respiratory syncytial virus, toxoplasmosis, cytomegalovirus and leptospirae were negative on at least two occasions. A Paul Bunnell test was negative as were tests for $HB_sAg$ and $HB_sAb$. Antibodies against smooth muscle antigen, mitochondrial antigen and parietal cell antigen were not found, a LE cell preparation was negative as were test for cold agglutinins and a direct antiglobulin test. The Rose-Waaler reaction was weakly positive; the AST was 1,000 U/ml. Blood, urine and throat cultures gave no bacterial growth, a skin test for tuberculosis was negative.

An X-ray of the thorax was normal. A skin biopsy showed infiltration of the dermis, epidermis and vascular walls by lymphocytes, a picture consistent with toxic allergic dermatitis. Immunofluorescent studies disclosed granularly deposited IgM in the blood vessel walls.

A bone marrow aspiration, taken 3 days after admission, showed apart from some pro-erythroblasts hardly any erythropoiesis (fig. 1). The granulocytopoiesis

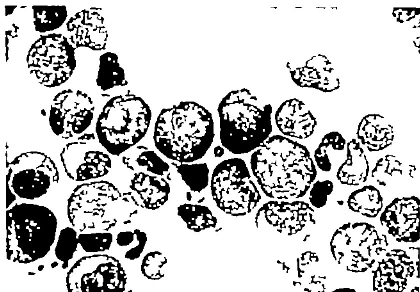


*Fig 1* Representative picture of the bone marrow specimen taken 3 days after admission. Scarcely some proerythroblasts and hardly any other erythropoetic cells are visible.

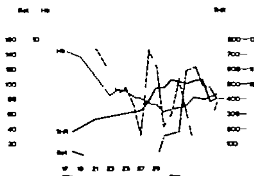



*Fig 2* Haematological data from November 17 till December 7. Hb = Haemoglobin concentration in mmol/l; Ret = reticulocyte count in ‰, THR = platelet count in 10⁹/l; L = [illegible] blood cell count in 10⁹/L

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nign lymphadenopathy [8, 9] HYMAN and SOMMERS [10] suggest a mitogenic effect of the drug.

The patient under discussion suffered from a transient pure red cell aplasia. Thymoma is implicated in 30–50% of cases of pure red cell aplasia and several infections may cause self-limiting erythroid aplasia [7], but neither syndrome seemed to be present in our patient. Therefore, and because of the striking temporary relationship, toxicity due to diphenylhydantoin seems the most likely explanation for the transient pure red cell aplasia in our case.

Two reports in the literature associate diphenylhydantoin and pure red cell aplasia [4 11] In one report two patients are described who suffered from a transient period of pure red cell aplasia starting during diphenylhydantoin treatment and rapidly disappearing after cessation of the drug. Both patients received corticosteroids but the benefit of these drugs in treatment of diphenylhydantoin induced pure red cell aplasia is not apparent from this report [11] The second report mentions a patient to whom diphenylhydantoin was repeatedly administered, each occasion leading to a course of self-limiting pure red cell anaemia [4] In a more recent report on the same patient YUNIS *et al.* [20] made it likely that diphenylhydantoin exerts its toxic effects by specifically inhibiting DNA synthesis in erythroid cells.

Because of ethical reasons we were unwilling to prove or disprove the connection between pure red cell anaemia and diphenylhydantoin treatment in our patient by readministering the drug. This report stresses the relation between diphenylhydantoin and a variety of haematological abnormalities, pure red cell aplasia being a rarity

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was shifted to the left but otherwise normal, megakaryopoietis was normally present. The haptoglobin, serum folate and vitamin B , levels were within the normal range.

In the first 9 days after admission the haemoglobin fell to 6.1 mmol/l, the reticulocyte count to zero while the platelet count remained normal and the leucocyte count above 6.4 × 10⁹/l (fig. 2) On November 29 after being absent for 3 days, the reticulocyte count rose to 0 6 and within 6 days to 12%. The haemoglobin rose to normal values in 3 weeks.

Bone marrow aspiration, taken on December 4 showed a normal erythropoiesis. The lymph nodes and skin rash waned within 5 days after admission. After application of diphenylhydantoin on the skin on December 12 the lymph nodes became enlarged for some days and the skin rash reappeared. Skin application of phenobarbital or coffein elicited no reaction.

The patient is well 2¹/₂ years after the observation and no haematological abnormality is apparent.

## *Discussion*

In our patient it seems most likely that the lymph node enlargement and skin rash present at the time of admission were due to diphenylhydantoin considering the fact that cutaneous application of this drug later on caused a reappearance of these symptoms. The patient was ill and noted lymph node enlargement before taking the drug and this period was probably related to a viral infection. After taking the drug the lymph nodes became tender and more enlarged At the time of admission no infectious cause of these symptoms could be determined despite elaborate investigations

Lymph node enlargement during treatment by diphenylhydantoin may occur in the absence of other manifestations of a drug reaction, but is usually associated with fever eosinophilia skin rashes, hepatic and splenic enlargement and blood dyscrasias [3] Symptoms may start as soon as 1 week after commencement of therapy or may be delayed for as long as 2 years. The lymph node enlargement may occur anywhere on the body but is most frequently located in the cervical region [15] Usually it disappears rapidly after cessation of the drug [15] In the literature there are several reports on patients who developed lymph node enlargement during diphenylhydantoin treatment which turned out to be caused by malignant lymphoma [1 10 13] The differential diagnosis between malignant lymphoma and benign lymphadenopathy in patients taking diphenylhydantoin has proved to be difficult [6 8] Some patients developed malignant lymphoma months to years after a transient period of apparently be

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# Occurrence of Haemoglobin H in Leukaemia a Further Case of Erythroleukaemia

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*Key Words* Haemoglobin H Leukaemia Erythroleukaemia Red cell precursors Stem cell Clone

*Abstract.* Erythroleukaemia in an elderly Caucasian male was associated with the presence of 15% of haemoglobin H (Hb-H Hb-$\beta_4$) in the haemolysate, identified by electrophoretic analysis, isolation and 'finger-printing. The peripheral blood picture was dimorphic, with 40% of hypochromic and morphologically abnormal red cells. Inclusion bodies indicative of the presence of Hb-H occurred in 30% of the red cells after supravital staining. The rare occurrence of Hb-H in leukaemic conditions and its distribution in the red cells is discussed in relation to the possible clonal origin of leukaemia and the involvement of red cell precursors.

An unstable haemoglobin component, associated with red cell inclusion bodies after supravital staining with Brilliant Cresyl Blue, was found in several cases of erythroleukaemia by White *et al.* [34] In a case of chronic myeloid leukaemia (CML), an electrophoretically similar unstable haemoglobin was identified by Beaven *et al* [5] as haemoglobin H (Hb-H Hb-$\beta_4$) by isolation and finger-printing of the globin. Other reports of the occurrence of Hb-H in myeloproliferative syndromes [8, 14] and in erythroleukaemia [1 25] have since appeared for review see Bradley and Ranney [9] A further instance of this apparently rare disturbance of haemoglobin synthesis in adult myeloid leukaemia is reported here, occurring in an elderly male with erythroleukaemia.

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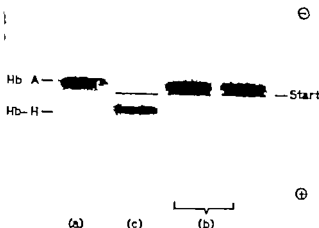



*Fig 3* Starch gel electrophoretic analysis of haemolysate- phosphate buffer system, pH 6.9. Normal adult haemolysate (a) subject' haemolysate (b), and Hb-H isolated from haemolysate of patient with Hb-H disease (c). Stained with Naphthalene Black.

liferation of erythroblasts, myeloblasts, myelocytes and monocytoid cells, megaloblastiform features were also present. Erythroleukaemia was diagnosed.

The blood reticulocyte preparation showed 7% of reticulocytes, and 30% of red cells containing fine greenish inclusions, suggesting the presence of Hb-H (fig. 4), so the haemolysate was studied

The anaemia was controlled by blood transfusions He had chronic bronchitis, and bronchopneumonia developed, his condition worsened and death occurred 4 months after admission. At post-mortem there was evidence of right ventricular hypertrophy pulmonary congestion, chronic bronchitis and bilateral upper-lobe fibrosis from old tuberculosis, venous congestion of liver spleen and kidneys was present. The marrow was hyperplastic and leukaemic, with predominance of myeloblasts, pro-myelocytes and monocytoid cells; erythroblast hyperplasia was less pronounced than 3 months earlier

## *Haemoglobin Studies*

A haemolysate prepared 10 weeks before death showed a fast haemoglobin component with the electrophoretic mobility of Hb-H at pH 8.6. At pH 6.9 this component had the same anodic mobility as authentic Hb-H isolated from a subject with Hb-H disease (fig. 3 4). No Hb-Bart's

## Methods

Standard haematological methods were used. Haemolysates were prepared without freezing in order to minimise loss of Hb-H. Starch gel electrophoretic analyses were made at pH 8.6 in Tris-EDTA borate buffer and at pH 6.9 in a phosphate buffer [17]. Published procedures [3, 24] were used to detect and estimate small proportions of fetal haemoglobin (Hb-F). Transparent starch gels stained with Naphthalene Black 10B were evaluated quantitatively by transmission densitometry [13]. The Hb-H fraction was isolated by electrophoresis in starch block in pH 8.6 barbitone buffer and the haem-free globin 'finger-printed' [2].

## Case History

A 72 year-old Caucasian male had a prostatectomy at age 62, and during the following 3 years his haemoglobin level was 12.7–13.0 g/dl. 6 years later a mixed, partly hypochromic blood picture was found with haemoglobin 8.6 g/dl and white cell count 6,100. 1 The haemoglobin fell to 4.4 g/dl over the next 9 months in spite of intramuscular iron therapy and he was admitted to hospital. A dimorphic blood picture persisted, with 60% of orthochromic red cells and 40% of hypochromic target, elongated and irregular cells (fig. 1) and leucoerythroblastic features: WBC 5,800 with 4% myelocytes, 81% polymorphs and 0.2% normoblasts; serum folate 3.2 ng/ml; vitamin $B_{12}$ 365 pg/ml. Marrow aspiration showed hyperplasia, with pro-




*Fig. 1.* Peripheral blood film, showing dimorphic pattern of orthochromic and hypochromic red cells. May-Grünwald-Giemsa. × 800. Length bar = 10 µm.

*Fig. 2.* Peripheral blood film after supravital staining with Brilliant Cresyl Blue showing inclusion bodies in a proportion of the red cells. × 1,100. Length bar = 10 µm.

bility with various myeloproliferative syndromes which may all be essentially neoplastic [26, 28]

In view of the patient's earlier clinical and haematological history the final imbalance of α-chain and β-chain synthesis was likely to be acquired, in association with the erythroleukaemia. The presence of free β-chains, due to relative depression of α-chain synthesis in the affected cells [14] during progressive depression of total haemoglobin synthesis, resembles some other cases of erythroleukaemia [1 25 34] CML and myeloproliferative syndromes [8, 14] The findings in these adult cases are in contrast to the high levels of Hb-F in certain juvenile chronic and acute myeloid leukaemias [4 29] A further distinction between the abnormalities of red cell components in the juvenile and adult cases is that in the former the levels of Hb-$A_2$ and carbonic anhydrase B are often very low as normally found only during the first few months of life: this finding lends support to the suggestion [29] that in juvenile CML there is a reversion to the fetal pattern of haemoglobins and red-cell enzymes, and that the condition may involve proliferation of a stem-cell line which does not differentiate normally to give the adult pattern. In the present case the Hb-$A_2$ level was normal, and the carbonic anhydrase B was elevated.

Eight cases with Hb-H and leukaemia or myeloproliferative disorder referred to above, the present case and two cases of severe anaemia and Hb-H with no obvious genetic basis [7] have all occurred in middle-aged or elderly Europeans. By contrast, the subjects described by BRADLEY [8] and WHITE *et al* [33] came from ethnic groups where hereditary Hb-H disease may occur [18]

10 weeks before death of the present case the red cell population contained excess β-chains, appearing in the haemolysate as Hb-H (15 ): this proportion is within the range found in previously reported leukaemias (up to 40%) The dimorphic blood picture showed 40% of hypochromic abnormal red cells, whilst the inclusion bodies formed after supravital staining with Brilliant Cresyl Blue were confined to 30% of red cells. It seems likely therefore, that the excess of β-chains is contained in less than half of the red cell population. In Hb-H disease itself the distribution of inclusion bodies and hence of Hb-H in the red cells is also not uniform [27] but virtually all the cells are hypochromic. In the present case of erythroleukaemia, however the findings strongly suggest that the disturbed balance of globin chain synthesis is confined to a particular fraction of precursor cells, as originally suggested by BEAVEN and WHITE [6]

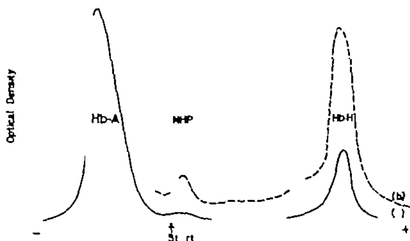



*Fig 4* Transmission densitometry record of starch gel electrophoretic analysis of patient's haemolysate phosphate buffer system, pH 6.9 stained with glycerol based Naphthalene Black. — — Blue filter — red filter minor zones on anodic side are benzidine-negative non-haem proteins (NHP)

Hb-$\gamma_4$) was seen and Hb-F could not be detected The Hb-H was 15% of the total haemoglobin, Hb-$A_2$ was 2 9% and the principal non-haem protein (carbonic anhydrase B) was 3 1% (referred to total haemoglobin)

The finger-print of the Hb-H component showed only $\beta$-chain peptides, confirming its identification as Hb-H

*Discussion*

The relationship between Di Guglielmo's disease (erythraemic myelosis) and erythroleukaemia, and the need to distinguish the former from thalassaemia and refractory anaemias, has been discussed by DAMASHEK and BALDINI [10] In the present case no relatives were available, but there was nothing to suggest the presence of a familial anaemia. Marked erythroblastaemia was not seen in spite of initial marrow erythroblast hyperplasia, but the terminal cytological evolution resembled that described by HINDMARSH and WICKHAM [15] in erythraemic myelosis terminating in erythroleukaemia, with myelomonocytic cells suggestive of Naegeli para myeloblastic leukaemia Myelomonocytic morphology of leukaemic leucocytes is well recognized in erythroleukaemia, as well as interconverti

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Direct experimental evidence in support of this view has now come from studies [1, 20, 22] of erythroleukaemia in a 66-year-old French male, showing that the associated Hb-H was confined to a distinct population of hypochromic red cells with a variety of biochemical, antigenic and structural abnormalities as compared with the co-existent population of normal red cells. The two populations were separated by exploiting their differences in density. These findings are entirely in accord with our present inference that only the hypochromic cells contain Hb-H and therefore constitute a separate population of abnormal erythroid cells.

The possible single-cell origin of a variety of malignant haematological disorders has frequently been considered. The distribution of the Ph chromosome in CML suggested [31] that erythroid, granulocytic and megakaryocytic cells all derived from a common ancestral stem cell in which the chromosome abnormality had appeared. Subsequent work has supported the monoclonal origin of human leukaemia, particularly in CML [12]. Very recently WEATHERALL and coworkers [32] have described a case of leukaemia with very high proportions of both Hb-H (60%) and of highly hypochromic red cells (98%). They showed that despite the severe repression of $\alpha$-chain synthesis the $\alpha$-globin genes were normal, and therefore suggested that in the leukaemic cell line there was a defect in the control of $\alpha$-globin gene transcription.

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# Auto-Immune Haemolytic Anaemia Complicating Infectious Mononucleosis in a Patient With Hereditary Elliptocytosis

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*Key Words.* Haemolytic anaemia · Hereditary elliptocytosis · Infectious mononucleosis · Erythrophagocytosis

*Abstract.* Auto-immune haemolytic anaemia complicating infectious mononucleosis occurred in a patient with hereditary elliptocytosis. A cold antibody of IgM anti-I specificity with narrow thermal amplitude was identified in the serum and the erythrocytes were found to be coated with complement. Significantly excessive erythrophagocytosis was demonstrated in samples of the patient's blood which had been chilled and then incubated at 37 °C. The patient recovered spontaneously. The elliptocytosis does not appear to have contributed to the episode of haemolytic anaemia: the other elliptocytic member of the family (her father) has no history and no present evidence of haemolysis.

## *Introduction*

Haemolytic anaemia is an uncommon complication of infectious mononucleosis. It may be due to auto-antibodies, hypersplenism, inherited red cell defects aggravated by the disease or, less likely, 'the retention of a metabolic toxin' [1, 11]. The present report documents a case of auto-immune haemolytic anaemia complicating infectious mononucleosis in a patient with hereditary elliptocytosis. Erythrophagocytosis which has previously only been postulated for such cases [16] has been demonstrated *in vitro* in this case. This process may well have been promoted by IgM anti-I.

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# Auto-Immune Haemolytic Anaemia Complicating Infectious Mononucleosis in a Patient With Hereditary Elliptocytosis

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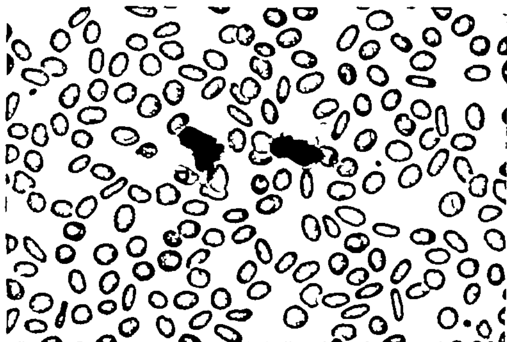


*Fig 1* Elliptical red cells and 'glandular fever' cells are shown. Leishman's stain × 700.

## *Case Report*

A girl aged 12 years, previously in good health, was seen by her general practitioner with a 2 week history of sore throat, malaise and jaundice. Preliminary examination of her peripheral blood showed elliptocytosis and clumping of her red cells, Hb = 11.3 g/dl, PCV = 0.312, reticulocytes = 3.2% and a leucopenia of 2.8 × $10^9$/l with 40% 'glandular fever' cells (fig. 1). The patient and her family were investigated 6 days later. Physical examination of the patient showed prominent, bilateral, cervical and axillary lymphadenopathy, a palpable spleen and an inflamed throat. The blood findings at that time were: Hb = 10.9 g/dl, PCV = 0.307, reticulocytes = 5.6%, platelets = 165 × $10^9$/l and white cell count = 9.3 × $10^9$/l with persistence of numerous 'glandular fever' cells. The bilirubin was 22.5 μmol/l, plasma haptoglobins < 250 mg/l and urobilinogen was found in the patient's urine. The Mono-Spot Test was positive. The Paul Bunnell Reaction was positive to a titre of 1/448 and the antibody was completely absorbed by ox cells, but not by guinea pig kidney. The alkaline phosphatase was 17.7 K. A units and the γGTP was 14 IU. Autohaemolysis was slightly increased after 48 h incubation at 37 °C. The osmotic fragility and the incubated osmotic fragility were normal. Ham's test and Donath Landsteiner Reaction were negative. 3 months later the patient had recovered spontaneously; her red cells are still elliptical.

## *Materials and Methods*

Blood from the patient, her parents and sister were processed in Coulter 'S' counter and films examined. Examination for elliptocytosis included wet preparations as well as dry films. The former were made by diluting whole blood 1 part in 200 with autologous plasma, as advocated by Goodall *et al.* [8]. The ABO and rhesus blood groups were determined by standard techniques [6].

Semiquantitative direct antiglobulin tests were performed using serial dilutions of broad-spectrum and specific antiglobulin sera [6]. Titres were expressed as the greatest dilution that produced definite agglutination. Investigation for IgG anti-I, in the presence of IgM anti-I was carried out by inactivating the IgM antibody with 2-mercaptoethanol [14].

Erythrophagocytosis was demonstrated by Goodall [7] modification of the method of Zinkham and Diamond [17]. In this simplified technique, freshly taken, heparinised venous blood was incubated in clean Wintrobe tubes for 1 h at 37 °C. The tubes were then centrifuged at 3,000 rpm for 8 min. The buffy coat was made into 2–4 smears and the erythrophages (monocytic and polymorphonuclear) were counted against 10,000 potential phagocytes.

Normal, group O circular red cells and the elliptical red cells of the patient and her father were examined for agglutination by the patient serum. All red cells were washed three times in 0.9% saline and incubated for 2 h at 4 °C, with doubling dilutions of the patient's serum.

## *Results*

The Coulter 'S' counts and indices on samples of blood from the relatives were normal. The ABO and rhesus groups and the presence of elliptocytosis are shown in figure 2. The findings are compatible with the observed linkage between the genes for elliptocytosis and the rhesus blood group system (elliptocytosis and CDe). Because of the lack of close relatives, extensive family studies were not possible.

### *Tests for Auto-Antibodies*

The direct antiglobulin test was positive to a titre of 1/80 positive to anti-complement serum, but negative to anti-IgG serum. The patient's serum contained a cold auto-antibody with the strongest reaction against cord cells of a normal, newborn infant (table I). Negative reactions were obtained following treatment of the patient's serum with 2-mercaptoethanol. The antibody would therefore appear to be an *IgM anti-I*.

| | | |
|---|---|---|
| ♂ (36 years) | X | ♀ (36 years) |
| O CDe cde (elliptocytosis) | | A CDe cde |
| ♀ (patient) | | ♀ (9 years) |
| O CDe cde (elliptocytosis) | | O cde cde |

*Fig 2* Family's ABO and rhesus groups and elliptocytosis.

*Table I* Titres, thermal range and specificity of cold agglutinin

| | Gp. O adult cells | Patient's own cells | Gp. O cord cells |
|---|---|---|---|
| 4 °C | 1/16 | 1/16 | 1/128 |
| [illegible]1 °C | – | 1/[illegible] | 1/16 |
| 37 °C | – | – | 1/4 |

*Table II* Erythrophagocytosis with and without prior chilling

| Test heparinised blood | Erythrophages/10,000 phagocytes (based on 2 counts/subject) | |
|---|---|---|
| | incubated for 1 h at 37 °C | chilled at 4 °C for 15 min then incubated for 1 h at 37 °C |
| Patient | 10.0 ± 2.0 | 75 0 ± 4 0 |
| Normal controls (5) | 1.8 ± 1 6 | [illegible]0 ± 1.3 |
| Infectious mononucleosis patients without haemolysis (4) | [illegible]3 ± 1 3 | 1.3 ± 0 9 |
| Father | 4 0 ± 2.0 | 3 0 ± 2.0 |

*Susceptibility of Elliptical and Circular Red Cells to Agglutination with Patient's Serum*

Normal, group O circular red cells and the elliptical red cells of the patient and her father were agglutinated to a titre of 1/16 by the patient's serum

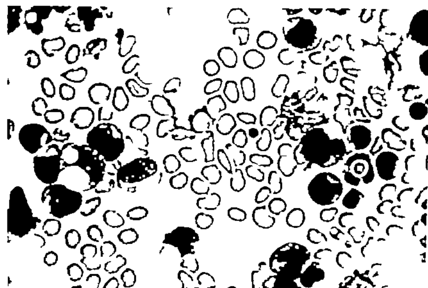


*Fig 3* A monocyte erythrophage is shown on the left and neutrophil erythrophage on the right from an incubated sample of the patient blood previously chilled. Leishman stain 700

*Erythrophagocytosis*

According to GOODALL [7], a score of more than 10 erythrophages per 10 000 potential phagocytes is significantly abnormal for fresh, heparinised blood, incubated at 37 C for 1 h His data were obtained from 100 normal controls and a range of patients with haemolytic anaemia.

In my experiments (table II) blood from 5 normal controls, 4 patients with infectious mononucleosis without haemolysis and the father showed normal scores for erythrophages, both with and without prior chilling.

The patient s blood without prior chilling gave a borderline figure but when chilled before incubation, there is a massive increase in the erythrophage score Erythrophages, both monocytic and neutrophil, are shown in figure 3

*Discussion*

Haemolytic anaemia occasionally complicates infectious mononucleosis. HOAGLAND [9] stated that 3°/ of his 500 West Point cadets developed

auto-immune haemolytic anaemia. The pathogenesis of the haemolytic anaemia has not been precisely defined. Since JENKINS *et al* [10] reported a wide thermal amplitude cold agglutinin of anti i specificity in a case of haemolytic anaemia complicating infectious mononucleosis, a large number of similar cases have been reported [3 12–15] However not all of the antibodies have been anti i BRAFIELD [2] described a patient with an auto-antibody which agglutinated both I and i cells to the same titre at 4 °C.

The anti-i may be IgM (19S) or IgG (7S) CAPRA *et al* [4] found an IgG anti i in 90% of his patients and postulated that an interaction between IgM anti IgG and IgG anti i is responsible for the auto-immune haemolytic anaemia In the present case no IgG anti i was demonstrable. This finding is similar to that of WILKINSON *et al* [14] whose 3 patients did not have an IgG anti i but had an IgM anti-i

WILKINSON *et al* [14] have emphasized the thermal range of anti i. They have stated that in many cases the thermal range was too low to be of importance in the pathogenesis of the haemolysis. The precise temperatures to which the blood falls in extremities exposed to cold are difficult to assess. However CLARKE *et al* [5] using thermo-couples and placing their patients arms in a water-bath at 1 C, estimated that the muscle temperature and the blood temperature in the arterioles was 17 C. In measurements of fascial tissue within 1 cm from the surface, they obtained temperatures between 10 and 15 C, and immediately below the skin temperatures were between 6 and 10 C. This degree of exposure to low temperature is rather extreme but it is possible that very much lower temperatures than the 28–32 C suggested by WORLLEDGE and DACIE [16] may occur in the small blood vessels of the skin in wintry conditions. In the present case, the patient s illness started in February and she was frequently outdoors

Erythrophagocytosis was not observed in thin films or buffy coat preparations made from freshly taken sequestrene blood. However because of the rapid removal of erythrophages by the reticulo-endothelial system it is only in extreme examples that non incubated blood shows this phenomenon It is therefore necessary for identification and quantification to incubate heparinised samples [17]

Significant erythrophagocytosis was demonstrated *in vitro* in monocytes and neutrophils. According to GOODALL [7] neutrophil erythrophages imply that there is high concentration of antibody and/or complement coating the red cells. GOODALL [7] further suggested that red cells

damaged by immune or other processes are ingested most avidly by reticulo-endothelial cells, less so by blood monocytes and least by neutrophils. Thus, the presence of erythrophagocytosis by neutrophils may indicate an even more intense phagocytosis by the spleen and other reticulo-endothelial sites. The promotion of such phagocytosis in these warm sites may have been initiated by binding of complement by the IgM anti-I in the relatively cold dermis.

In certain diseases where there is a hereditary defect in the red cell membrane, there is an increased agglutinability by anti-I. In a review of the literature, WORLLEDGE and DACIE [16] recorded 5 such cases, 3 of hereditary spherocytosis and 2 of thalassaemia. There has been no documentation of such a case with hereditary elliptocytosis. The present investigation does not suggest that there is increased agglutinability by anti-I in hereditary elliptocytosis.

Therefore, the reaction with anti-i and thus presumably the haemolysis in this patient appears due to the infectious mononucleosis and the elliptocytosis was co-incidental. The experiments on erythrophagocytosis suggest that this process may well have been of importance in the pathogenesis of the haemolysis.

## *Acknowledgments*

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# Defective Maturation of Granulocytes, Retinal Cysts and Multiple Skeletal Malformations in a Mentally Retarded Girl

A New Syndrome

(With 1 colour plat )

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The Copenhagen Ey Clinic for the Mentally Retarded, Gentofte

*Key Words.* Monosegmented granulocytes · Mental retardation · Adducted thumbs · Micromelia · Microphthalmia · Retinal non-attachment

*Abstract.* A case of congenital malformations of the extremities (deformed thumbs and great toes, dislocation of the hips, limitation of motion of the joints of the lower extremities), bilateral microphthalmia, bilateral retinal cysts, cerebral atrophy epilepsy severe physical and mental retardation and monolobed neutrophil granulocytes is reported. A similar clinical picture has not previously been de scribed. W assume that the patient suffers from sublethal genetic disorder

A patient with malformations of the extremities, microphthalmia, reti noschisis, leucocytic anomalies, cerebral atrophy epilepsy and mental re tardation has been observed for 3 years. The combination of these clinical manifestations represents an as far as known undescribed syndrome. The patient concerned was mentioned by UNDRITZ [1] under the designation of Plum s syndrome, and by WARBURG [2] but the case history has not previously been published.

## Case Report

The patient is the youngest sibling f 7 the 2nd, 3rd and 6th pregnancies terminated in spontaneous abortions in the 3rd month of gestation. 2 sisters and 1 broth-

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*Fig 1* Pneumoencephalography 18 months old, severe cerebral atrophy

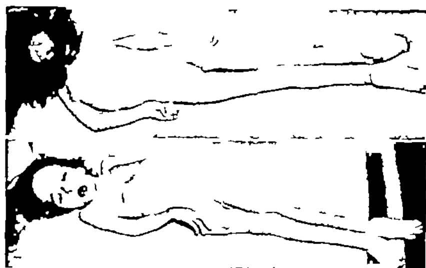


*Fig. 2.* Photograph of the patient showing chest deformity and abnormal dentition

er are normal. The parents are unrelated, and there is no familial predisposition to similar manifestations. The mother was 43 years at the birth of the patient.

The pregnancy was uncomplicated, the infant was delivered in breech presentation, birth weight 2,550 kg length 49 cm The Apgar scores were 2 immediately on birth, and 8 at 8 min A large amount of fluid was aspirated from the airways, and cardiac massage was given immediately on birth The baby was placed in an incubator for 8 days. Multiple skeletal malformations were observed at birth.

During the first 3 years of life, the girl was repeatedly admitted to various hospitals, where congenital hip dislocations, malformations of the hands and feet, bilateral microphthalmia, retinoschisis and epilepsy were observed. At the age of 18 months, pneumo-encephalography revealed severe cerebral atrophy (fig. 1)

The girl failed to thrive, showed severe physical and mental retardation, and had frequent episodes of upper respiratory infection. At the age of 8 months, seizures developed, and treatment with phenemal was instituted.

*Physical examination* At the age of 38 months, physical examination revealed small, slightly built girl (weight 9.5 kg, length 83 cm) with severe mental retardation. The skull was lengthened and flattened, circumference 51 cm. The ears were low-set, the forehead prominent Hypertelorism was present. The mouth was small and triangular the hard palate high and the lower jaw very small. Prognathism, with about 1 cm retraction of the incisors of the lower jaw was recorded The dentition was slightly irregular (fig 2).

The chest was asymmetrical (fig. 2), the sternum short, and there was slight dextroconvex scoliosis The joints of the upper extremities were loose, without hyperextension. The thumbs were adducted along the palmar surfaces, the other fingers and

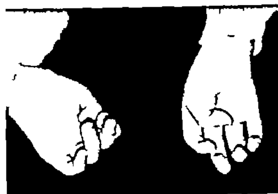
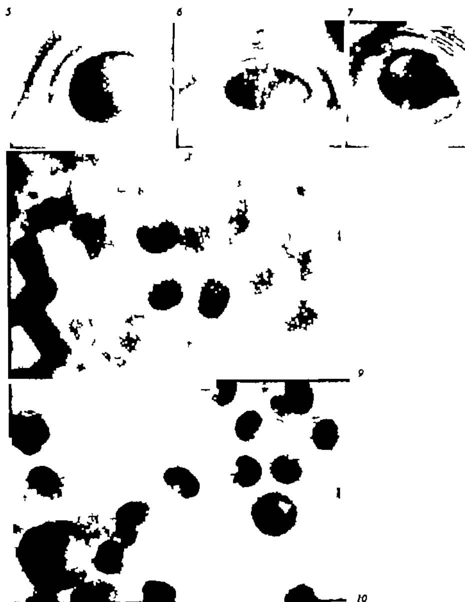


*Fig 3* Right hand. The thumb is adducted



*Fig 4* Dislocation of the great toe.

the nails showed normal development distal palmar flexion creases were present on both hands (fig. 3) The pelvis was small and narrow The hip joints were flexed. There was incomplete passive hip extension maximum flexion 70° on the right and 80° on the left side. The rotation of the hip joints was markedly restricted. Shortening (? cm) of the right femur was noticed the lower legs were of equal length. The knees were flexed, with limitation of both extension and flexion (range of motion 170–130° on the right and 160–100° on the left side) The feet were short, plump, turned outwards and held in equinus position the range of motion of the ankle joints was only about 10° The great toes and first metatarsi were short the toes de




*For legends see overleaf*

*Fig 5* Right eye. The most anterior part of the pigmentary retrolental cyst is seen at 9–10 o clock. The vascular network from the top of the cyst running into the vitreous is just visible.

*Fig 6* Right eye. The deeper parts of the cyst occupy nearly one half of the inner wall of the eye. It is multiloculated, but does not appear solid.

*Fig 7* Left eye. An abortive coloboma is seen in the iris at 3 o clock. Behind this area, a dark cyst of the posterior epithelium of the iris is protruding into the pupil and into the vitreous further back. Fine capillaries project from the cyst, forming a loose network. The iris is markedly pale blue.

*Fig 9* Typical neutrophil granulocytes with round nuclei from the peripheral blood.

*Fig 10* Bone marrow from the patient showi g abnormal development of the granulocytes.

rotated laterally (fig. 4). Cl mealth be kin, heart and bdominal iscera were normal.

*Neurological examination.* The fac l musculature tak, but symmetrical. The girl did not respond t acoust stimuli. She could hold up her head, but could not sit up without support. When held the erect position, she did not attempt to stand. *The musculature was h potonic and soft. There ere no pareses. The deep* reflexes were ery weak or absen. The grasping reflex was preserved. No response to plantar stimulation.

*Ophthalmological examination.* The child appeared blind and did not f llow light with the eyes. The corneal diameter w 10 mm in both eyes. The distance between the internal canthi as 33 mm, that between the external canthi 80 mm.

Both pupils responded to light nd consensually they ere slightly dislocated temporally. The left iri showed coloboma t 3 clock (fig 7). In th temporal quadrants of both retinae large cysts ere visible (f g 5–7). They xtended from the equator to the pupillary margin, involving the pigment epithelium f the iris and Zinn circle. Retinal vessels were seen on the surface of the cysts, from which they extended as arcades of fine vessels towards the centres of the itreous. There was no connective tissue around these vascular loops, nor any haemorrhages or abnormal pigmentations in the fundi. The optic nerves ere atrophic.

*Electro-encephalography.* Repeated studies showed severe or moderately severe abnormalities, with frequent occurrenc of slow wa es with changing laterality *and of spikes and sharp wes over the left hemisphere.*

*Radiography f skeleton.* Severe dislocation of both hips with considerable upward displacement was disclosed. The feet were held in equinus position with proximally dislocated great toes (fig 8). The bones were slender presumably halisteretic, with delayed osseous development. In particular centres of ossification were absent in the patellae. The bones of the skull, spine and chest were normal.

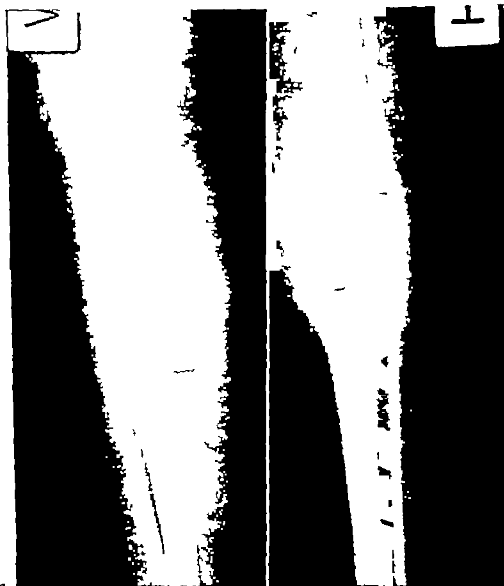
*Laboratory investigation.* Differential counts of the peripheral blood showed 34–61 neutrophil granulocytes with round nuclei. Some of these cells had filaments radiating from the nuclei (table I, fig. 9).

Examination of the bone marrow showed that all myeloblasts were normal, with normal indentation of the nuclei. During maturation the nuclear density increased, and at the same time the nuclei assumed round form, with fine filaments presenting at one pole (fig 10).

The leucocyte count, haemoglobin level, erythrocyte count, sedimentation rate, the serum levels of creatinine, sodium, calcium, phosphorus and chloride, and the examination f urine all showed normal findings, and so did electrocardiography.

*Clinical course. During* the 3-year period of observation at the Kolonien Filadelfia, the osseous malformations remained unchanged and the retinoschisis did not *progress. No mental development occurred during this period, and the physical* growth was very modest. At the age of 6 years, the girl cannot sit up, stand, hear or see. She has acquired no ocal sounds. Body weight 11.1 kg, length 95 cm. She has had few epileptic seizures and is continually under treatment with phenemal.

The blood picture has remained largely unchanged during the observation period. Neutrophil polymorphonuclears are practically absent, and ll neutrophil granulocytes contain round nuclei (table I).



8a

*Fig 8.* Radiographs of the extrem ties showing very thin, presumably halisteretic bones. The feet are in eqn nus position. The metatarso-phalangeal joint of the great toes are dislocated. The musculature is very gracile: the patellae are absent. Assessed by the method of Greulich, the bone ages are dissociated: the carpus corresponds to slightly less than 1 months, the finger bones to 30 months. (The radiographs were taken when the patient was 3 years old.)

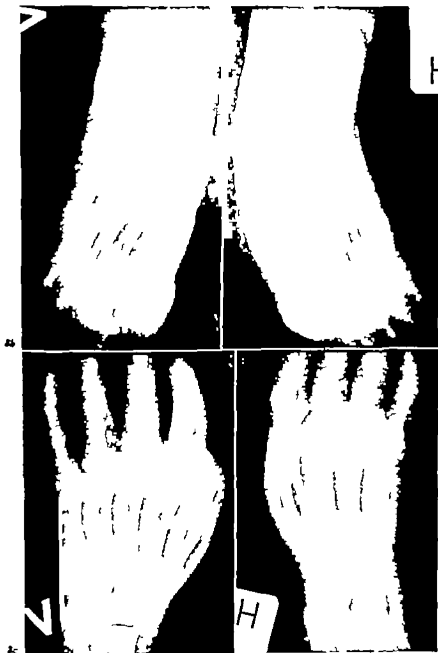

8b
8c

*Table I.* Differential counts of peripheral blood from the proband (H.H.)

| | February 1 1973 | March 27 1973 | May 3 1973 | April 24 1974 | May ~7 1974 | December 12, 1974 | April 29 1975 |
|---|---|---|---|---|---|---|---|
| Neutrophil granulocytes[1] | | | | | | | |
| Staff cells | 34 | 5? | 50 | 55 | 43 | 61 | 51 |
| Segmented cells | – | – | 1 | 2 | 1 | 1 | 0 |
| Eosinophil granulocytes | 6 | 5 | 0 | 5 | 9 | 3 | 6 |
| Basophil granulocytes | 1 | – | – | – | – | 1 | – |
| Monocytes | 3 | 9 | 4 | 7 | 7 | 3 | 4 |
| Lymphocytes | 56 | 34 | 45 | 31 | 40 | 31 | 39 |

[1] For the distribution of nuclear lobes, see table III.

*Study of the family.* A comprehensive study of the blood groups and HLA genotypes of the proband's parents and of the healthy siblings was performed (table II). The paternity was not in doubt. The karyotypes of the patient, 2 of her siblings and the parents were normal. The segregation of the fluorescence markers of chromosomes 1, 2, 4, 9, 14, 15, 21 and 22 followed the usual pattern. As seen from table III, blood smears from the parents and 3 siblings all showed a normal distribution of the nuclear lobes (Arneth's classification).

## Discussion

The first study of blood smears from our patient aroused suspicion of the Pelger-Huët anomaly. However, in this condition the blood picture usually reveals about 30% monolobed granulocytes [3] as contrasted with 96–98% in our patient. We then thought that the patient might be homozygous for the Pelger-Huët anomaly, but as the blood pictures of the parents were normal this hypothesis had to be abandoned. Homozygosity for the Pelger-Huët anomaly associated with osseous malformations has been observed in rabbits [4]. However, skeletal or ocular malformations have not been described in human beings with the Pelger-Huët anomaly, either in the ordinary heterozygous form or in the three homozygous cases reported in the literature [5, 6].

Although all her granulocytes were monolobed, we do not believe that our patient suffers from the Pelger-Huët anomaly proper, since bone mar

*Table II* Blood groups and HLA genotypes of the proband (H.H.), her parents and two siblings (July 1973) the paternity is not in doubt

| Relationship | Date of birth | HLA genotype | Chromosomes | ABO | Other blood groups nd antibodies |
|---|---|---|---|---|---|
| Father J Aa. H | July 18, 1925 | HLA A9 B8/Aw19 B14 | b | B | cde/ dc P MNss, Le( b ) K Fy( + ) Lu( ) |
| Mother I H | May 9 1927 | HLA-A.., Bw15/B27 Cw1 | d | A | CcDee P MNss, Le( + b ), K Fy( + ), Lu( ), Dr1 |
| Sister K. H. | October 25 1956 | HLA-A9 B8/A.., Bw15 | ac | A B | de,cde P MNss, Le( + b ), K Fy(a + ), Lu(a ) |
| Brother F H | March 18, 1958 | HLA Aw19 B14/A.., Bw15 | bc | A | C Dee, P MNss, Le( + b ), K Fy( ), Lu( ), Dr1 |
| Proband H. H. | February 15, 1970 | HLA A9 B8/A2, Bw15 | ac | A | C Dee, P MNss, Le(a + b − ), K − Fy( + ), Lu( ), Dr1 |

*Table III* Arneth's classification showing shifts to the left in the number of lobes of the neutrophil granulocytes in the proband (H H ) and the corresponding counts in her parents 3 siblings and 25 control persons

| Relationship | Date of birth | Number of lobes 1 | | 3 | 4 | 5 or more | total |
|---|---|---|---|---|---|---|---|
| Proband H H. | February 15 1970 | | | | | | |
| February 1 1973 | | 98 | 2 | | | | 102 |
| April 4 1974 | | 96 | 3 | 1 | | | 105 |
| April 29 1975 | | 97 | [illegible] | 1 | | | 104 |
| Father J An. H | July 18 19[illegible]5 | 10 | 28 | 45 | 14 | 3 | [illegible] |
| Mother J H. | May 9 19[illegible]7 | 1 | 26 | 48 | 1 | [illegible] | 266 |
| Sister [illegible] H | October [illegible]5 1956 | 5 | 20 | 55 | 16 | 4 | 294 |
| Brother F H | March 18 1958 | 8 | 31 | 44 | 14 | 3 | 273 |
| Sister J S. H | September 24 1951 | 7 | [illegible]6 | 49 | 14 | 4 | 28[illegible] |
| Normal controls (n = 25) | | 5 | [illegible]3 | 49 | 20 | 3 | 293 (266–317) |

row examination showed that the cells right from the metamyelocytes had a normal configuration with indented nuclei. During the maturation the structure changed into a round nucleus with filaments at one pole, as it is described in the Stodtmeister type of the Pelger Huët anomaly [6 7 10] Thus in our patient, the nuclear structure was morphologically similar to that of the mature granulocytes seen in the Pelger Huët anomaly whereas the immature cells were normal

In 1967 Baratta [11] reported 2 cases of neutrophil R anomaly in which the patients had up to 43% neutrophil granulocytes with round nuclei. However during the observation period, the frequency of these cells decreased from 43 to 26% within 1 week The 2 patients were unrelated and had no congenital malformations.

The advanced age of the mother at the birth of our patient is presumably irrelevant as an aetiological factor as she had previously had three spontaneous abortions in the 3rd month of gestation If these fetuses had the same disease as our patient, the disorder might be due to a sublethal genetic mutation

*Differential diagnosis* To the best of our knowledge, cases similar to that reported here have not previously been published. In Larsen's syndrome [8] with congenital dislocations, the ophthalmological changes ob-

served in our patient were absent, and in patients with congenital retinal non attachment similar physical and mental defects have not previously been described [9]

## *Acknowledgements*

The authors wish to express their gratitude to the staff of the Queen Louise Children Hospital for the permission to publish the radiographs. Our thanks are also due to Dr Margaret Mikkelsen, the Kennedy Institute, Glostrup, for performing the chromosome examinations and to Dr F Kissmeyer, Blood Bank and Blood Grouping Laboratory Arhus Kommunehospital, for the HLA and blood grouping determinations.

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# Book Review Buchbesprechung Livre nouveau

V. TILENER und W. GRFUL. Hämatologie und Hämostasiologie. UTB Band 340. Steinkopff Darmstadt 1975. XII + 107 pp. 21 fig. 19 tab. DM 17.80.

Das Büchlein besteht aus einem ersten methodischen und einem zweiten deskriptiv klinischen Teil. In beiden Teilen versuchten die Autoren, durch Auslassen von nicht primär relevantem Material die Kürze der Darstellung zu wahren. Bei kritischer Durchsicht ist das Resultat eher enttäuschend. Ein Beispiel mag genügen. Ich zitiere aus Abschnitt 12, p. 15 «Sternalpunktion»: «Diese Untersuchung ist in der Praxis nur bedingt durchführbar. Sie erfordert Übung, ist dann allerdings keine für den Patienten belastende Methode.» Soll auch der Medizinstudent der späten siebziger Jahre nichts von der Gefahr einer Sternalpunktion hören? Ist das Sternum wirklich der Ort der Wahl für eine Knochenmarkspunktion?

Ein kurzes Regi ter darf insgesamt als nützlich bezeichnet werden. Von mässiger Qualität sind die Abbildungen. Auch der geübte Hämatologe wird Mühe haben, auf Abbildung 6 die erwähnten »Megaloblasten« zu finden (Sternalmark bei perniziöser Anämie). Ein fleissiges, aber in vielen Einzelheiten zu verbesserndes Büchlein!

E. A. BECK, *Bern*

## Errata

In the article 'Classic Hemophilia A in a Female' published in Vol. 58, No. 4, pp. 94–102, 1977 an unfortunate error has occurred on page 94. The name of the last-named author is incorrect and should read STANFORD T. ROODMAN.

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Acta haemat. 59 65–72 (1978)

# Evaluation of the Usefulness of Serum γ-Glutamyl Transpeptidase Levels in the Management of Haematological Neoplasia

B. E. Roberts, J. A. Child, E. H. Cooper, R. Turner and J. Stone

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Department of Experimental Pathology and Cancer Research, University of Leeds,
and Regional Radiotherapy Centre, Cookridge Hospital, Leeds

*Key Words.* Leukaemia · Lymphoma · γ-Glutamyl transpeptidase · Liver function

*Abstract.* The levels of serum γ-glutamyl transpeptidase (GGT) and, when appropriate, alkaline phosphatase (AP) and 5'-nucleotidase (NTD) have been measured as a routine in 276 patients with malignant haematological diseases during a 26-month trial period. GGT levels add no prognostic information to the routine haematological surveillance of leukaemia. Polychemotherapy does not appear to be an inducer of liver drug-metabolising microsomal enzymes. Polycythaemia rubra vera, myelofibrosis and chronic lymphocytic leukaemia may cause little change in GGT, AP and NTD levels despite marked hepatomegaly. A raised GGT in Hodgkin's disease and non-Hodgkin lymphoma is generally associated with active and widespread disease, but not necessarily a sign of malignant tissue in the liver. The elevations of GGT in myeloma may be secondary to liver infiltration though this group merits further detailed study.

There has been a growing interest in using measurements of serum γ-glutamyl transpeptidase (GGT) levels as a warning system for the presence of metastatic cancer in liver [1, 7–10]. A rise of GGT tends to precede changes in other markers of hepatic cell membrane disorders such as 5'-nucleotidase (NTD), alkaline phosphatase (AP) and the transaminases [12].

As infiltration of the liver may be found in all types of haematological

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As infiltration of the liver may be found in all types of haematological

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neoplasia [9] it was decided to screen all patients attending our clinics so that experience could be obtained in interpreting the results of GGT and where appropriate comparison was made with concurrent levels of NTD and AP

## *Patients and Methods*

This investigation was commenced in August 1974 and completed in October 1976. The 276 studied patients were adults with leukaemias, lymphomas, immunocytomas or myeloproliferative diseases attending out-patient clinics and had blood taken for GGT estimations at the time of their routine blood counts. The duration of surveillance was from 2 to 26 months. The stage of evolution of the disease in the individual patients varied considerably from first presentation to terminal phases. In analysing the data, the broad experience of the whole population of patients within each major disease group is considered first, and then the sub-sets within the diseases are analysed with respect to stage and clinical course. All patients with Hodgkin's disease were staged clinically and pathologically according to the Ann Arbor classification [4]

### *Liver Function Studies*

GGT (EC 2.3.2.2) was estimated by JACOBS method [6]. NTD (EC 3.1.3.5) was determined according to VAN DER SLIK *et al* [11] AP (EC 3 1.3.1) was assayed by the technique of BESSEY *et al.* [3]. The upper limits of normal values (mean $\pm$ 2 SD) for these enzymes were as follows. GGT 30 IU/l NTD 15 IU/l AP 60 IU/l.

## *Results*

The frequency of elevation of serum GGT in malignant haematological disease can be seen in table I this table records the proportion of patients in each disease group who have had one or more GGT values in the moderate (31–100 IU/l) or marked (> 100 IU/l) range of elevation In the patients with GGT > 100 IU/l the AP was > 60 IU/l in 75% and the NTD > 15 IU/l in 64%. In all the patients, except in 2 cases of myeloma and in 1 of chronic myeloid leukaemia, elevations of GGT > 200 IU/l were accompanied by coincidental rises of AP and NTD When there was a rise of GGT in the moderate range the AP and NTD showed an irregular pattern of disturbance many of the patients having these two serum enzymes within normal limits.

### *Hodgkin's Disease*

The 50 patients with Hodgkin's disease included all stages and histopathological types. All 6 patients with markedly elevated GGT had stage

*Table I* Distribution of GGT values in malignant haematological diseases

| Disease | Number of patients | Serum GGT < 30 IU/l | 31–100 IU/l | > 100 IU/l |
|---|---|---|---|---|
| Hodgkin's disease | 50 | 35 | 9 | 6 |
| Non-Hodgkin lymphomas | 26 | 12 | 7 | 7 |
| Myeloma | 22 | 11 | 7 | 4 |
| Acute myeloid leukaemia | 22 | 12 | 7 | 3 |
| Acute lymphoblastic leukaemia | 15 | 11 | 3 | 1 |
| Chronic granulocytic leukaemia | 25 | 11 | 3 | 11 |
| Myelofibrosis | 12 | 5 | 4 | 3 |
| Polycythaemia rubra vera | 71 | 45 | 22 | 4 |
| Chronic lymphocytic leukaemia | 33 | 25 | 6 | 2 |

Includes patients who have not been followed since the start of their disease.

IIIb or stage IV disease. Of the 9 patients with moderately raised GGT 3 had advanced disease (IIIb or IV) 1 of these had died and 1 is not in clinical 'remission Generally patients with GGT < 30 IU/l had earlier less aggressive, disease with 3 exceptions (more advanced than IIIa) all but 2 patients (who have been lost to follow-up) out of 35 are alive and currently in clinical remission.

*Non-Hodgkin Lymphoma*

Out of 26 patients 7 had a high GGT value, all of whom had evidence to suggest extensive and very aggressive disease 3 have been classed as histiocytic lymphomas 4 patients have died and only 1 patient is currently clinically disease-free. 1 of the group had obstructive jaundice. All 6 patients with moderately increased GGT levels could be regarded as having extensive, diffuse disease (all histiocytic) 2 have died and only 1 is clinically disease-free. Only 3 of 14 patients with GGT < 30 IU/l had histiocytic lymphomas, the remainder had, generally less aggressive disease the majority of patients are in clinical remission with no evidence of disease.

*Myeloma*

In our series of 22 patients 4 had GGT > 100, 2 of whom had advanced disease (1 was terminal with a leuco-erythroblastic blood picture) both had considerable numbers of circulating plasma cells. This contrasts with the other 2 patients: 1 with an apparently non-secreting solitary plasmacytoma, and 1 with a solitary bony lesion and modest paraprotein-

aemia both had AP and NTD levels within normal limits. Of the 7 patients with moderately raised GGT 1 died with evidence of very extensive disease and 2 others appear to be deteriorating rapidly as judged by clinical features and immunoglobulin studies.

At least 2 of the patients with normal values had advanced disease (1 has died) and it is not possible to discern any obvious clinical or pathological differences between these sub-groups. Of note is the fact that 2 patients who appear to have reached an accelerated phase of the disease had normal GGT levels initially but have subsequently developed moderately raised levels.

*Acute Leukaemia*

In both acute myeloid and acute lymphoblastic leukaemia there was no correlation between the initial GGT value and the incidence of remission A rise of GGT terminally was seen in some patients but it was not an inevitable feature of the disease.

The patients with acute lymphoblastic leukaemia received vincristine and prednisolone to induce remission and combinations of 6-mercaptopurine, methotrexate, vincristine, prednisolone, cyclophosphamide, asparaginase cytosine arabinoside and adriamycin during their maintenance therapy Patients with acute myeloid leukaemia received daunorubicin, cytosine arabinoside and thioguanine, and in some cases drugs included in the list above except for asparaginase. This list indicates the potential hepatotoxic agents used in managing these patients. However despite this, no patients showed progressive rise in GGT levels during remission maintenance therapy

*Myeloproliferative Disorders*

*Polycythaemia rubra vera* 71 patients were investigated It was found that 37% had a level of GGT > 30 IU/l The raised GGT levels were not related to the clinical features of the disease or particular types of therapy Further study revealed that 10 of the patients were being treated for recurrent congestive heart failure and 8 of these had persistently elevated levels of GGT Longitudinal studies showed that in some patients the GGT levels increased slowly over 1–2 years.

*Myelofibrosis*

The distribution of GGT levels suggested that it was not until a later stage that the marked histopathological changes in the liver resulted in a

*Table II* Features of chronic myelocytic leukaemia patients with a consistently raised GGT >100 IU/l

| Case No. | Ph₁ chromosome | Splenectomy | Slow metamorphosis |
|---|---|---|---|
| 1 | neg. | 0 | 0 |
| 2 | neg. | + | 0 |
| 3 | pos. | + | + |
| 4 | pos. | + | + |
| 5 | pos. | 0 | + |
| 6 | pos. | 0 | 0[1] |
| 7 | pos. | + | + |

[1] Busulphan-wasting disease.

disturbance of liver function, and when it occurred, the physical signs of hepatosplenomegaly were out of proportion to the mild biochemical disturbances of liver function. The 3 patients with high levels of GGT were being treated for congestive heart failure.

### *Chronic Myeloid Leukaemia*

The pattern of change was dissimilar to the acute leukaemias being typified by a slow progressive increase of the GGT levels during the latter part of the disease and in particular during slow metamorphosis especially after splenectomy However other patterns of disease may be associated with a rising GGT (table II) The level of GGT did not have any direct relation to the patient's well-being, levels of > 500 IU/l were compatible with being able to work. When the rate of change of the level of GGT is rapid, it is closely associated with an exacerbation of the disease and the expected symptoms of ill health. 2 patients developed acute blast cell transformation whilst under continuous surveillance without elevation of the serum GGT

### *Chronic Lymphocytic Leukaemia*

Although hepatomegaly was evident clinically in more than half the patients with chronic lymphocytic leukaemia listed in table I it appeared to cause very little disturbance of liver function as indicated by the distribution of GGT values.

*Discussion*

Evidence of disturbed liver function as indicated by changes in GGT may well prove valuable both in the initial assessment and subsequent monitoring of patients with malignant lymphomas and possibly immunocytomas. There is considerable evidence that abnormalities in liver function tests do not invariably mean that the liver contains identifiable Hodgkin's tissue, for quite often biopsy will indicate either the presence of granulomata or non-specific change in the liver. Conversely, as is well known, where biopsy is performed as part of a staging routine, Hodgkin's tissue may be demonstrated in some patients who have normal liver function tests [2, 5]. However, in our patients with Hodgkin's disease, a high GGT appeared to be usually associated with advanced disease. Conversely, the majority of patients with stages I–IIIa disease generally have consistently normal levels; a GGT level < 30 IU/l may be accordingly regarded as indicating a relatively good prognosis. Although the number of patients is smaller, a comparable pattern has emerged in the non-Hodgkin lymphomas with an apparently greater tendency to raised GGT levels. This may possibly be related to the fact that the disease is frequently diffuse with an overall greater possibility of hepatic involvement. Staging is, however, considerably more complex than in Hodgkin's disease. It was of interest that in patients with histiocytic lymphomas there was a high incidence of raised GGT (9/12). Our overall experience is that GGT is more sensitive than AP and NTD for monitoring patients with lymphomas. Our current interpretation is that consistent elevation of GGT, especially in a patient who is known to have had GGT levels that were previously in the normal range, is indicative of progressive disease. However, on this evidence alone, it is not possible to say whether the liver is definitely infiltrated or reacting to the presence of malignant cells not recognisable as Hodgkin's tissue in the liver or, less likely, to some humoral agent produced in patients with active Hodgkin's disease. We believe that the sequential measurement of GGT could be used as one of an array of tests to monitor the patients once the diagnosis has been established, the disease staged and therapy instituted.

In the myeloma group, half the patients had GGT values > 30 IU/l at some time. Out of the patients with markedly raised GGT values, 2 appeared to have localised plasmacytomas (1 now definite myelomatosis) in contrast to the other 2 who had extensive diffuse disease, probably with hepatic infiltration. The infiltration of the liver by relatively few plasma

cells may cause disproportionate response from the GGT in the hepatic cell membranes it is also possible that myeloma cells may produce humoral factors which can act in the same way as *microsomal enzyme inducer* drugs. This needs further investigation.

In practical terms, although considerable disturbance of liver function, as represented by rise of GGT AP and NTD may occur in the leukaemias, knowledge of the levels of these enzymes does not appear to be of potential value in influencing clinical management or as a prognostic factor A possible exception would be changes which could be attributed to drug-induced hepatotoxicity and this, with the exception of a case of Busulphan toxicity appeared to be rare.

The serum GGT is now well recognised to be a marker of the effects of various compounds such as barbiturates and anti-convulsants that induce drug-metabolising enzymes in liver microsomes [8] However the pattern of elevation of GGT in the malignant haematological disorders is far too sporadic to suggest any causal relationship between its elevation and drugs used for cancer chemotherapy This is particularly true in the adult acute leukaemias which, despite intense polychemotherapy may show a normal GGT throughout their illness. It is more reasonable to believe *that an elevation of GGT in our patients with malignant haematological* disorders, which the few exceptions mentioned above, is probably the result of cellular infiltration of the liver or conceivably a response to a humoral agent produced by the malignant cells.

## *Acknowledgements*

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# False Positive Acidified Serum Test in a Preleukemic Dyserythropoiesis

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*Key Words.* Acidified serum test · Preleukemia · Dyserythropoiesis

*Abstract.* In a case of preleukemic dyserythropoiesis, *in vitro* red cell lysis tests showed a positive acidified serum test whose characteristics are described. The positive acidified serum test occurred in 10 normal sera, in 1 serum with complete deficiency of the fourth component of complement and in 1 serum with complete deficiency of the second component of complement. The test was found negative with 2 hyperlipemic sera. The other *in vitro* red-cell lysis tests were negative. The results of the *in vitro* lysis tests were different from the results obtained in paroxysmal nocturnal hemoglobinuria and congenital dyserythropoietic anemias.

## *Introduction*

In paroxysmal nocturnal hemoglobinuria (PNH) the red cells are unusually susceptible to lysis by complement. Lysis of PNH red cells may be obtained *in vitro* in fresh serum where the complement system is activated either through the classical pathway (cold antibody lysis test) or the alternate pathway (acidified serum test sensitized with magnesium, inulin test, cobra venom test) or both pathways (acidified serum test sensitized with thrombin, sucrose lysis test) [4, 11, 12].

The acidified serum test (Ham's test) is widely used to detect the PNH abnormality and generally considered to be specific for this red cell defect

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[4] However this test is also positive in congenital dyserythropoietic anemia type II or HEMPAS [3] but, in contrast to PNH, HEMPAS red cells undergo lysis in only a portion of normal sera and they do not undergo lysis in the patient's own acidified serum and the sucrose lysis test remains negative A positive acidified serum test has also been observed in 2 cases of congenital dyserythropoietic anemia type I [1 14] but in 1 case the positivity was obtained in heated sera and due to spherocytosis [1] A positive acidified serum test and a positive sucrose lysis test occurred also in 2 cases of lymphoproliferative disease [7 17]

The present report describes the characteristics of a positive acidified serum test in a case of preleukemic dyserythropoiesis.

## *Patient Summary*

A 27 year-old man without noticeable antecedents was admitted to the Hematological Clinic in January 1975 for an anemia and neutropenia discovered in September 1974 Physical examination revealed a paleness and no other abnormalities. Laboratory findings showed hemoglobin 11 g/100 ml RBC 3.32 million/mm³ WBC 3,600/mm³· leukocyte differential count 19% neutrophils, 1% basophils, 6% monocytes, 73% lymphocytes and 1% neutrophilic metamyelocytes reticulocytes 33‰. The peripheral blood showed anisocytosis, anisochromia, Howell Jolly bodies, Cabot rings and basophil stippling. Platelet count 210,000/mm³ The bone marrow was markedly hypercellular with 70% erythroblasts and numerous erythroblastic islands. On light microscopy a small population of polychromatic and orthochromatic erythroblasts showed abnormalities usually observed in congenital dyserythropoietic anemias: bi- or multinuclearity incomplete cell divisions. Electron microscopy confirmed the light-microscopic findings: there were erythroblasts with two nuclei or bilobulated nuclei, protrusion of nuclear material into the cytoplasm. Some erythroblasts showed a marked irregularity and 'spongy' appearance of the nucleus with wide nuclear pores and 'invasion of the cytoplasm into the nucleus. The characteristic morphological feature of the cytoplasm was the presence of abnormal cisternae delimitated by a membrane located near the cell surface. Deposits of iron were often found in the enlarged nuclear pores, in the mitochondria and in the zone of adherence of two adjacent erythroblasts [15].

Hemoglobin study revealed an increased level of HbF (6%) Serum iron was 110 μg/100 ml total bilirubin 0.7 mg/100 ml. Iron-kinetic study indicated an hyperactive erythropoiesis. The curve of cell incorporation of labelled iron showed a fall at the 4th day indicating an early destruction of some newly formed red cells. However clinical or biological signs of intravascular hemolysis could not be detected.

The patient's group was 0 rhesus CcDee, Kell – with no irregular antibodies. Serum proteins showed a normal electrophoretic pattern. The acidified serum test was positive with the patient's serum and the serum of several different normal donors. The characteristics of this positive acidified serum test will be given thereafter

There was increased I and I antigen on the patient's red cells (agglutination titers with known anti-I serum: patient 1/16, adult control 1/2, cord control 1/256; with an anti-I serum, patient 1/2,058, adult control 1/128, cord control 1/32).

A study of the red cell enzymes showed an increased level of hexokinase, glyceraldehyde 3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glutathione peroxidase, while the level of phosphohexose isomerase was reduced at 50% of the normal level (Prof. P. Boivin). This defect was not observed in the other family members. The karyotype analysis showed no abnormality.

The blood picture remained stable without specific treatment until March 1976. At that time Hb was 10 g/100 ml, WBC 5,000 with 16% neutrophils, 31% monocytes, 42% lymphocytes, 4% neutrophilic myelocytes and 7% myeloblasts. Platelet count was 101,000/mm³. The anemia, thrombocytopenia and neutropenia persisted and worsened and the myeloblast percentage increased to a level of 53 in the peripheral blood and 28 in the bone marrow in November 1976 where the patient died, 24 months after the discovery of the anemia and 8 months after myeloblasts were first noted in the blood.

## *Materials and Methods*

Veronal-buffered saline (VBS) containing 0.00015 M calcium and 0.0005 M magnesium ions, VBS containing 0.01 M ethylenediaminetetraacetic acid (EDTA), disodium salt (EDTA VBS) and Alsever's solution were prepared according to MAYER [13]. VBS-sucrose buffer (60% isotonic sucrose-40% VBS) was prepared as outlined by LOOTS *et al.* [11].

Blood from the patient and from normal donors was collected in an equal volume of Alsever's solution and stored at 4 °C until used. Normal compatible serum was obtained from donors and stored at –30 °C for a short period of time. Serum from a patient with total genetic deficiency of the fourth component of complement and from a patient with total genetic deficiency of the second component of complement were used [5, 6]. Anti-I antibody was obtained from a patient with chronic cold *agglutinin syndrome*. Inulin was purchased from Prolabo (Paris), cobra venom factor was purchased from Cordis Lab. (Miami, Fla.).

The acidified serum test sensitized by the addition of magnesium (0.005 M) was performed according to MAY *et al.* [12]. The cold antibody test, sucrose test, inulin test and cobra venom test were performed according to LOOTS *et al.* [11]. Optimal concentration of inulin, cobra venom factor and cold antibody were defined in preliminary tests using AET-treated red cells [16]. Normal cells were not lysed in any of these procedures at the concentrations of reactants used.

## *Results and Discussion*

The results of the *in vitro* red cell lysis are given in table I. The acidified serum test was markedly positive with the patient's own serum, with

*Table I* Results of *in vitro* red cell lysis tests in the patient

| Test | Lysis, % |
|---|---|
| Acidified serum | |
| Patient's own serum | 47 |
| Normal donor serum | |
| 1 | 51 |
| 2 | 55 |
| 3 | 44 |
| 4 | 60 |
| 5 | 57 |
| 6 | 70 |
| 7 | 45 |
| 8 | 45 |
| 9 | 70 |
| 10 | 51 |
| 11 (hyperlipemic) | 0 |
| 12 (hyperlipemic) | 0 |
| C4-deficient serum | 45 |
| C2-deficient serum | 43 |
| Sucrose lysis | negative with all sera |
| Cold antibody lysis | negative with all sera |
| Inulin | negative with all sera |
| Cobra venom | negative with all sera |

the serum of 10 normal donors, with the C4-deficient serum and with the C2-deficient serum. The test was negative with the serum of 2 normal donors and it was observed that these sera were turbid, i.e. hyperlipemic. The test was negative with all sera heated at 56 °C for 20 min. The other *in vitro* red cell lysis tests remained negative with all sera. The tests were performed on three occasions (March 1975, April 1975 and March 1976) and gave similar results.

The positivity of the acidified serum test with the patient's own serum and the negativity of the cold antibody test excluded a congenital dyserythropoietic anemia type II (HEMPAS). The negativity of the insulin test, sucrose test and cobra venom test excluded an unusual susceptibility of the patient's red cells to lysis by complement as observed in PNH. Despite the fact that the acidified serum test was found negative after heating the sera at 56 °C for 20 min, there is little reason to believe that the complement system was involved in the lysis of the patient's red cells be-

cause the lysis was obtained with complement-deficient sera and because all the tests except the acidified serum test based on the activation of the complement system through the classical and/or alternate pathway remained negative. Therefore, we considered the positive acidified serum test as 'false positive'. The negativity of the acidified serum test with two hyperlipemic sera suggests that an excess of lipids or lipoproteins protected the patient's red cells against lysis and that the abnormal susceptibility to lysis in the acidified serum was due to an abnormal lipid composition or to an abnormal lipid metabolism in the patient's red cells. This hypothesis could not be verified.

In congenital dyserythropoietic anemia type I, a positive acidified serum test was observed in 2 cases [1, 14]. In 1 case [1] the lysis was obtained in 9 of the 10 normal acidified sera and with the patient's own acidified serum. However, hemolysis was marked only with 1 of the tested sera and lysis did not seem to depend on the presence of complement since sera heated at 56 °C for 30 min produced the same degree of lysis as unheated fresh serum. Therefore, the positive acidified serum test was also considered to be 'false positive'.

In PNH the evolution toward an acute myeloblastic leukemia is rare. Three observations have been published in 1969 [8–10]. The acute myeloblastic leukemia occurred 3, 7 and 2 years, respectively, after the discovery of the clinical and biological signs of PNH. The diagnosis of PNH was based on the recognition of episodes of hemoglobinuria, hemosiderinuria, on the positivity of the acidified serum test in the 3 cases and on the positivity of the sucrose test in 2 cases [9, 10]. In our observation we have not ascertained clinical or biological signs of PNH and the characteristics of the positive acidified serum test allowed us to exclude the diagnosis of PNH.

Catovsky *et al.* [2] have performed *in vitro* red cell lysis tests in several cases of acute myeloblastic leukemia and have found frequently a positivity of the cold antibody lysis test (58%) and less frequently a positivity of the sucrose lysis test, whereas the acidified serum test was never found positive. In the two cases of lymphoproliferative disease with a 'PNH-like' red cell abnormality reported [7, 17], the acidified serum test was positive in one case togehter with a positive sucrose lysis test [17] and positive in the other case together with a positive cold antibody lysis test and with a positive sugar lysis test [7].

Our observation of a preleukemic dyserythropoiesis confirms that a positive acidified serum lysis test is not sufficient to state a 'PNH defect'

in the red cells and that several tests are necessary to explore and characterize the *in vitro* susceptibility of red cells to lysis in the presence of normal sera, complement-deficient sera and also sera with abnormal lipid or lipoprotein composition.

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# Chimiothérapie d'induction des lymphomes malins non hodgkiniens

## Résultats d'un essai contrôlé comparant deux quadruples associations

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**Induction Chemotherapy of Non-Hodgkin Malignant Lymphomas. Results of a Controlled Trial Comparing two Quadruple Associations**

*Key Words* Non-Hodgkin malignant lymphomas Chemotherapy

*Abstract* Two types of chemotherapy have been compared in a randomized trial to treat non Hodgkin malignant lymphomas. 66 patients were included in this study but only 40 were evaluable after a histologic review of all cases. Treatment were an association of cyclophosphamide, vincristine, prednisone and doxorubicine or VM 26. Patients received only one induction chemotherapy course during 15–20 days. Results were evaluated immediately at the end of the course Side-effects were mild. Efficacy was about the same with the two protocols: 35 patients out of 40 experienced a remission of more than 50% (among them 8 experienced a complete remission) These results are better than previous ones obtained with cyclophosphamide, vincristine and prednisone only It is concluded that this type of treatment is well tolerated, quickly efficient and useful before treating patients with radiotherapy or long-term chemotherapy

Le traitement des lymphomes malins non hodgkiniens est essentiellement déterminé par le stade d'extension topographique Les stades I et II loco-régionaux avec extension lymphoïde exclusive d'un seul côté du diaphragme, relèvent ordinairement d une radiothérapie isolée qui permet de guérir près d'un malade sur deux tandis que les stades III et IV généralisés d une façon manifeste ou occulte, sont soumis à une chimiothérapie destinée à obtenir un résultat palliatif aussi bon et prolongé que possible.

A la suite des observations que nous avions faites dans les stades I

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et II pour la maladie de Hodgkin [5] nous nous sommes orientés depuis plusieurs années vers une association systématique radio-chimiothérapique dont les premiers résultats sont très encourageants [10]. Par ailleurs, pour les stades III et IV nous avions constaté dans un précédent essai contrôlé qu'il était utile de faire précéder la chimiothérapie d'entretien par un cycle court et énergique de chimiothérapie d'induction [4]. C'est dire l'importance que revêt pour nous la disponibilité d'une chimiothérapie brève aussi active que possible et bien tolérée pour ne pas compromettre la réalisation du temps thérapeutique ultérieur. Après deux études randomisées antérieures [2, 4] qui nous avaient permis de conclure à la prépondérance d'une triple association comportant cyclophosphamide, vincristine et prednisone, ce qui a été confirmé par d'autres équipes [3] nous avons amorcé un nouvel essai destiné à préciser l'utilité d'y ajouter une quatrième drogue en l'occurrence le VM 26 (CVPV) ou la doxorubicine (adriamycine CVPD), qui avaient été récemment introduits dans l'arsenal anticancéreux. C'est l'analyse de cet essai contrôlé que nous rapportons ici.

## *Malades et Méthodes*

*Malades traités.* Du 1er janvier 1974 au 31 août 1976, tous les malades adressés à la Fondation Bergonié avec le diagnostic de lymphome malin non hodgkinien et justifiant une chimiothérapie ont été inclus dans cet essai et randomisés pour recevoir l'un des deux protocoles de traitement. Au début de l'essai, seuls les malades de moins de 70 ans ont été inclus, puis en raison d'une excellente tolérance appréciée lors d'une exploitation préliminaire [1], tous les malades ont été inclus sans limite d'âge. 66 malades ont été ainsi randomisés avant d'être soumis à une chimiothérapie intégrée soit dans un protocole à visée curatrice pour les stades cliniques I et II, avant une radiothérapie, soit dans protocol palliatif pour les stades III et IV avant une chimiothérapie d'entretien plus légère. Tous ces malades ont fait l'objet du bilan préthérapeutique habituel avec examen clinique complet, radiographies thoraciques, lymphographie abdominale, biopsie ostéo-médullaire d'une crête iliaque et dosages biologiques usuels, il a jamais été fait de laparotomie exploratrice ni de ponction-biopsie du foie systématiques. Pour tous ces malades, le diagnostic histologique été soumis à une deuxième lecture avec colorations supplémentaires pour classification selon la nomenclature de Kiel [6]. Cett vérification conduit à éliminer 24 malades, 2 autres étaient exclus en l'absence de cibl tumorale objective *permettant de juger* convenablement de l'efficacité antitumorale: ces diverses causes d'exclusion sont précisées dans le tableau I pour tous les malades retenus pour cette analyse, le diagnostic de lymphome malin non hodgkinien pu être affirmé avec certitude. La distribution des principaux paramètres pronostiques est indiquée par le tableau II. On peut y observer que le premier groupe de malades recevant le proto-

*Tableau I* Causes d exclusion de 26 malades

| Causes d exclusion | | Nombre de malades |
|---|---|---|
| Document histologique non disponible | 3 | |
| Diagnostic histologique rectifié | 18 | |
| Carcinome anaplasique | | 7 |
| Autre carcinome | | 3 |
| Autre hémopathie maligne[1] | | 5 |
| Divers[2] | | 3 |
| Diagnostic histologique incertain | 3 | |
| Absence de cible objective | 2 | |

[1] Histiocytose maligne 2, myélome 1 leucémie myéloïde chronique transformée 1 lymphadénopathie angio-immunoblastique 1

[2] Sarcome d Ewing 1 réticulosarcome de Parker et Jackson 1 sarcome embryonnaire 1

cole CVPV est défavorisé, par rapport au second groupe avec un plus grand nombre de malades avec un type histologique défavorable, en rechute, ayant reçu un traitement antérieur et à un stade clinique IV avec atteinte viscérale.

*Protocole de traitement* Les deux protocoles utilisés diffèrent entre eux, d'une part, par l addition à la triple association de base comportant cyclophosphamide, vincristine et prednisone soit le VM 26 (CVPV) soit la doxorubicine (CVPD) et d'autre part, par l étalement des drogues espacées de 48 h dans le protocole CVPV et administrées simultanément dans le protocole CVPD (tab. III) L application de ce traitement s est faite soit chez des malades hospitalisés, soit après vérification de la bonne tolérance chez des malades ambulatoires à l'hôpital de jour Sauf exception, un seul cycle de ces protocoles a été appliqué et dans tous les cas c est l effet immédiat d'un cycle qui est rapporté. En dehors de la prednisone administrée par voie orale, tous les produits ont été administrés par voie veineuse en injection rapide dans une tubulure de perfusion

*Tolérance* Dans l ensemble, la tolérance aux deux traitements est bonne ou très bonne tant sur le plan clinique qu'hématologique. La chimiothérapie a été interrompue en raison de l apparition d'une leucopénie inférieure à 2000/ l chez 6 malades soumis au protocole CVPV et chez 1 malade recevant l association CVPD Aucune manifestation, en particulier vasculaire n a été relevée dans les deux groupes de malades. Aucun décès n a pu être rattaché au traitement.

## *Résultats*

Ils sont appréciés immédiatement à la fin de la chimiothérapie c est-à dire entre le 15e et le 20e jour selon le protocole avant le début d une

*Tableau II* Répartition des principaux critères selon les deux protocoles

| | CVPV | | CVPD | |
|---|---|---|---|---|
| Malades | | | | |
| Total | 22 | | 18 | |
| Hommes/femmes | 16/6 | | 14/4 | |
| Age > 60 ans | [illegible] | | 10 | |
| < 60 ans | 10 | | 8 | |
| Type histologique favorable | 10 | | 11 | |
| Lymphocytique | | 2 | | 2 |
| Centrocytique | | 1 | | 1 |
| Centroblasto-centrocytique | | 7 | | 8 |
| Type histologique défavorable | 12 | | 7 | |
| Lymphoblastique | | 5 | | 2 |
| Immunoblastique | | 7 | | 5 |
| Phase évolutive perceptible | | | | |
| n° 1 | 10 | | 13 | |
| n° 2 et + | 12 | | 5 | |
| Traitements antérieurs | | | | |
| Radiothérapie | 12 | | 3 | |
| Chimiothérapie | 7 | | 4 | |
| Stade clinique | | | | |
| I | 4 | | 4 | |
| II | 4 | | 4 | |
| III | 5 | | 8 | |
| IV | 9 | | 2 | |
| Présence de signes généraux (B) | 5 | | 3 | |

*Tableau III* Protocoles de traitement

| | Dose unitaire mg/m | CVPV | CVPD |
|---|---|---|---|
| Cyclophosphamide | 400 | jours 3, 10 et 17 | jours 1, 8 et 15 |
| Vincristine | 0,7 | jours 1, 8 et 15 | jours 1, 8 et 15 |
| Prednisone | 40 | du jour 1 au jour 15 | du jour 1 au jour 15 |
| VM 26 | 70 | jours 5, 12 et 19 | |
| Doxorubicine | 35 | | jours 1 et 15 |

*Tableau II* Appréciation de l'efficacité

| | CVPV n = 22 | CVPD n = 18 |
|---|---|---|
| Echec total ou partiel | 4 | 1 |
| Régression > 50 % | 13 | 14 |
| Rémission complète | 5 | 3 |

radiothérapie ou d une monochimiothérapie d entretien qui se situe dans tous les cas le 21e jour au plus tard. Le tableau IV indique la fréquence et l importance des régressions tumorales appréciées après ce bref délai. Il n existe aucune différence significative entre les résultats obtenus avec chacun des deux protocoles. Un taux d échec supérieur avec le protocole CVPV doit être rapproché de la distribution inégale des malades entre les deux protocoles en défaveur du CVPV En effet, tous les échecs observés avec le protocole CVPV sont survenus chez des malades avec atteinte vis cérale (stade IV) l échec noté avec le protocole CVPD s est traduit par le décès, 24 h après la mise en route du traitement, provoqué par une attein te tumorale très avancée L'homogénéité des résultats obtenus consistant dans trois quarts des cas en une régression incomplète supérieure à 50 %, ne permet de dégager aucun facteur pronostique influant sur la réponse thérapeutique En ce qui concerne le type histologique la régression tumorale est en général un peu plus rapide dans les formes défavorables mais le résultat apprécié en fin de chimiothérapie est comparable dans les deux groupes de malades (à histologie favorable ou défavorable)

Une rémission complète est rarement obtenue à la troisième semaine mais la régression tumorale presque toujours supérieure à 50 % du volume initial permet d aborder le second temps du traitement dans d excellentes conditions.

## *Commentaires*

Ces protocoles d association chimiothérapique s intègrent dans le cadre d un traitement plus complexe qui comporte ultérieurement soit une radiothérapie soit un autre type de chimiothérapie Ce second temps thérapeutique a pu dans tous les cas, être institué sans retard compte tenu de la bonne tolérance au premier temps chimiothérapique

Pour les stades cliniques I et II nous avons observé en effet que l'induction chimiothérapique initiale améliore les résultats obtenus par la radiothérapie en ce qui concerne aussi bien l'obtention d une rémission complète initiale que les resultats à long terme [10] ces effets notés pour les lymphomes non hodgkiniens sont comparables à ce que nous avions précédemment constatés pour la maladie de Hodgkin [5] Encore faut-il que la chimiothérapie initiale ne compromette pas la radiothérapie nécessaire: c'est le cas avec les protocoles utilisés qui sont suffisamment bien tolérés et à l issue desquels l'état des malades est en général très amélioré en raison de l effet bénéfique sur la tumeur ce qui leur permet d aborder la radiothérapie et de la supporter mieux que si elle avait été réalisée d emblée. Enfin, après l'irradiation, un nouveau cycle de chimiothérapie est appliqué, avec ou sans un intervalle libre d un mois selon l âge du malade, complétant ainsi une séquence chimio-radio-chimiothérapie en sandwich» avant que le malade soit laissé sans aucun traitement d'entretien. La qualité des résultats ainsi obtenus est d'autant plus remarquable que les malades sont soumis à un bilan préthérapeutique limité sans exploration chirurgicale et à un traitement court qui, globalement, n excède jamais 3 mois [10]

Pour les stades cliniques III et IV l effet favorable de la chimiothérapie initiale est en général encore plus appréciable notamment sur l'état général le passage à un traitement ambulatoire d'entretien s opère sans difficulté et permet à pratiquement tous les malades entrés en rémission de voir cette rémission se compléter en 1 ou 2 mois, ce qui constitue un délai souvent nécessaire à la rétrocession complète de tumeurs dont l'évolution spontanée progressive aussi bien que régressive, est souvent lente [9]

Ces observations sont difficiles à comparer avec les effets des chimiothérapies cycliques, appliquées sur le modèle du MOPP qui a fait ses preuves dans la maladie de Hodgkin, à raison d'un cycle par mois ou toutes les 3 semaines pendant 6 mois [3 8] Nos résultats globaux qui ont été publiés ailleurs [7] paraissent au moins aussi bons que ceux obtenus dans d'autres séries mais un choix entre deux conceptions thérapeutiques sensiblement différentes ne pourra être fait qu à l'issue d'un essai comparatif Nos observations actuelles permettent d'offrir une alternative à l attitude généralement suivie, alternative qui a pour elle de bons résultats et plus encore une excellente tolérance.

Ces résultats ne permettent pas de choisir entre les deux protocoles comparés qui apparaissent équivalents. En raison de sa simplicité d'admi-

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nistration à raison d une injection intraveineuse par semaine seulement (contre 3 pour le CVPV) un léger avantage se dégage en faveur du protocole CVPD Avec les réserves à émettre au sujet de toute comparaison de type historique, on constate que ces résultats d ensemble obtenus avec les deux associations quadruples sont nettement plus favorables que ceux que nous avions observés précédemment avec la triple association cyclophosphamide vincristine et prednisone administrée sous différentes formes et qui correspondaient à environ la moitié de régression tumorale supérieure à 50% [2, 4]

Enfin nous voudrions souligner l importance qu il y a de contrôler le diagnostic histologique pour des états pathologiques mal définis jusqu à ces dernières années et auxquels on pouvait associer d autres affections, comme les carcinomes anaplasiques dont le pronostic est évidemment très différent. Il est certain que le traitement de lymphomes non hodgkiniens bénéfice actuellement d un diagnostic histologique beaucoup mieux assuré

## Résumé

Un essai thérapeutique randomisé, comparant deux protocoles de quadruple association chimiothérapique dans le traitement des lymphomes malins non hodgkiniens, est analysé. Chaque association comportait cyclophosphamide vincristine et prednisone avec VM 26 ou doxorubicine La présentation de 40 traitements évaluables fait ressortir la qualité des résultats obtenus après 15–20 jours de traitement, avec une bonne tolérance. Il n'y a pas de différences significatives entre les résultats obtenus avec chacun des deux protocoles. De tels protocoles doivent s'intégrer dans une stratégie thérapeutique globale qui est brièvement discutée.

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CRICCA *et al.* [20] showed that administration of hydroxyurea (HU), a potent DNA synthesis inhibitor, induces extensive killing of marrow replicating erythroblasts in the S phase of their cell cycle. As a result of the severe cell destruction, these investigators showed that there is rapid commitment of stem cells to the depleted compartment. Furthermore, MARTIN *et al.* [15] reported on increased content of CFUs and CFUc in the bone marrow of mice administered five doses of HU over a period of 32 h.

In such extreme conditions of cell depletion, the question arises as to whether stem cells in the bone marrow are sufficient in number to replenish the depleted compartments, or stem cells of extramedullary origin need to be recruited.

In a previous study on rats, we found that administration of a single injection of HU kills approximately 50% of nucleated marrow cells within 9–10 h of the injection [3]. Differential counts of this hypocellular bone marrow disclosed a high percentage of lymphoid cells which was attributed to an actual inflow of lymphocytes in the range of 14,000,000 cells in one femur. Upon microscopic examination, such marrow appears predominantly lymphoid [?]. Implantation of the lymphoid-like marrow in peritoneal diffusion chambers in irradiated mice was conducive to enhanced growth of hematopoietic colonies [3]. These results suggest that the lymphoid marrow is enriched in stem cells.

The present study on rats attempts to investigate on possible origin of extramedullary stem cells which, as a result of HU-induced bone marrow damage, become committed to various blood cell lines. The peritoneal diffusion chamber (DC) technique was used to test the capacity of spleen cells of rats administered HU to induce growth of hematopoietic colonies. The results obtained indicate that the spleen of rats contains stem cells which become committed soon after the occurrence of severe depletion of young marrow type cells.

## *Material and Methods*

### *Donor Rat for Spleen Cells and the Mode of Preparation of the Cellular Suspension*

Male or female rats of the Hebrew University strain, weighing 100–120 g, were used as donors for spleen cells. The rats were administered a single intraperitoneal injection of HU (400 mg/kg body weight), and 1–3 h following the administration of the drug, suspension of spleen lymphocytes was prepared as follows: Pooled spleens of 3–4 rats were minced and further passed through a metallic gauze under sterile

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# Growth of Diffusion Chamber Hematopoietic Colonies Derived from Spleen Cells of Rats Administered Hydroxyurea[1]

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*Key Words* Rat spleen Diffusion chamber colonies Hydroxyurea Commitment of stem cells

*Abstract* Donor rats of the Hebrew University strain were administered a single intraperitoneal injection of hydroxyurea (400 mg/kg body weight). 1–3 h following the administration of the drug, a suspension of spleen cells, the majority of which consisted of lymphocytes, was prepared Spleen cells were placed in diffusion chambers and these were implanted in the peritoneal cavity of preirradiated mice. 5–8 days following implantation, erythroid and granulocytic colonies developed in 30.3% of the diffusion chambers studied. However in most chambers, macrophages were observed. In control experiments with implantation of spleen cells of normal rats, granulocytic colonies did not grow and in only 3 1% of the chambers erythroid colonies were noted. Macrophage colonies, however developed in all 32 control cultures.

Our previous studies showed that administration of a single dose of hydroxyurea strips the rat bone marrow of approximately 50% of replicating cells within 9–10 h. The results of the present study indicate that such a severe depletion of rat marrow cells results in early commitment of spleen stem cells to various blood cell lines.

Hematopoiesis is stimulated in experimental animals and in human beings by a reduction of the red blood cell volume as in hemorrhage and hemolysis or by an increased demand for leukocytes as in infections. Recently it was reported that a reduction of young type cells in the bone marrow may also stimulate hematopoiesis [11 16 18 21] In mice, REN-

[1] Hydroxyurea for this study was donated by the Squibb & Sons Inc. Company Princeton, N J USA, to whom the authors are very grateful.

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CRUCCA *et al.* [20] showed that administration of hydroxyurea (HU), a potent DNA synthesis inhibitor, induces extensive killing of marrow-replicating erythroblasts in the S phase of their cell cycle. As a result of the severe cell destruction, these investigators showed that there is rapid commitment of stem cells to the depleted compartment. Furthermore, MARTIN *et al.* [15] reported on increased content of CFUs and CFUc in the bone marrow of mice administered five doses of HU over a period of 32 h.

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conditions. The cells were collected in tissue culture medium M 199 containing antibiotics, and the suspension was then centrifuged at 900 rpm for 10 min. Cells from the buffy coat which consisted mainly of lymphocytes, were further washed in culture medium and a final suspension of 1–3 × 10⁶ cells/ml was prepared. This suspension was enclosed in DC made of plastic rings of 13 mm diameter and millipore filter paper of 0.2 μm pore size.

*Host Mice for Peritoneal DC*

28 male or female mice of the 'Sabra' strain weighing 20–25 g, were used as host animals. 4 h prior to implantation of the chambers in the peritoneal cavity the mice received total body irradiation of 550 r in a single fraction (170 r/min) at source-target distance of 50 cm, with a Picker Vanguard 280-kV X ray machine and filtration of HVL 1 mm Cu. Two chambers were inserted into the peritoneal cavity of each mouse. The hosts received regular food and water and were kept 2 in a cage. The DC cultures were studied at daily intervals from days 5 to 8 after implantation.

*Processing Technique of DC Cultures and Assessment of Colonies*

The cultures were processed according to a technique detailed in previous publications [3–6]. It is nevertheless important to mention here that the fibrinous clot was not dissolved by pronase but was processed as such for light and electron microscopy in the conventional way. Semithin sections from various areas of growth in each chamber were examined by light microscopy and presence or absence of colonies was noted. Upon previous experience examination of semithin sections can be used as a reliable method for establishing the occurrence of colonies in chambers [3]. Ultrathin sections of areas of colonies were stained by uranyl acetate and lead citrate and studied with a Philips 300 electron microscope.

*Control Experiment*

Spleen cells of normal untreated rats of the same strain and weight as those used for the experiments were implanted in DC of 16 preirradiated mice. The cultures of controls were processed as detailed above and studied 5–8 days following implantation.

## *Results*

### *Experimental Group: HU Treated Donor Rats*

A total of 56 chambers were studied by light microscopy for assessment of hematopoietic colonies. Among these there were 13 chambers in which erythroid colonies were observed, 2 chambers containing both erythroid and granulocytic colonies and 2 chambers in which granulocytic

*Fig. 1.* Area of erythroid colony in DC. Erythroblasts represent the large majority of cells. A few lymphocytes are also observed. Light micrograph. × 650.

*Fig. 2.* Cluster of erythroblasts in DC colony. The cells in this area are of basophilic and polychromatophilic types. Electron micrograph. × 7 150.

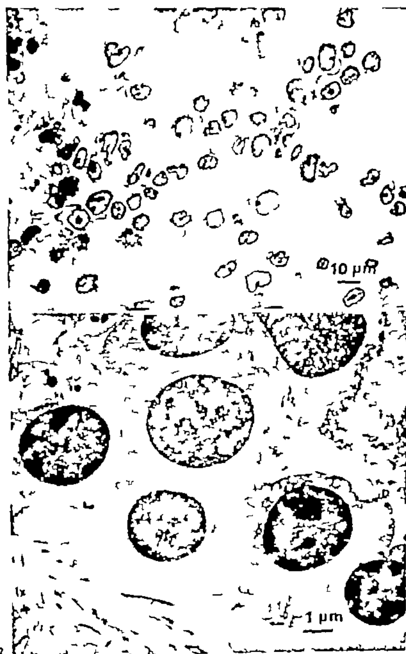

10 µm
1 µm
1
2




*Fig 3* Large granulocytic colony in DC. Cells of all degrees of maturation are noted Light micrograph × 600.

colonies developed Thus, in 30.3% of chambers, erythroid and granulocytic colonies were noted However in a large majority of the total number of chambers studied, macrophage colonies were observed Most erythroid and granulocytic colonies were seen on days 7–8 of the implantation

The erythroid colonies consisted of groups of 5–20 cells on examination by light microscopy (fig 1) By electron microscopy it was possible to observe erythroblasts in various stages of maturation (fig 2) Occasionally erythroblasts were seen in the close vicinity of macrophages which showed ingested material in their cytoplasm

The granulocytic colonies displayed a different pattern of organization consisting of large accumulations of cells, in the range of 100–200 cells per semithin section at a magnification of × 120 Such a growth pattern suggests that a single or only a few stem cells gave rise to the colony (fig 3). By electron microscopy cells ranging from myeloblasts to mature granulocytes were seen to possess characteristic and normal ultrastructural features (fig 4)

The macrophage colonies consisted of accumulations of groups of 2–3 macrophages or of single cells scattered in the fibrinous clot (fig 5) This




*Fig 4* Part of granulocytic colony from figure 3. Typical myeloblasts, myelocytes and mature granulocytes are observed. In between cells there are strands of fibrin. Electron micrograph 6,300

growth pattern highly suggests that each individual cell had matured from a young type precursor with or without a mitotic step in between. By electron microscopy the macrophages disclosed a large variety of organelles in the cytoplasm and short projections at the surface membrane, which are characteristic features for these cells (fig 6)

Other cell types such as lymphocytes, large lymphoblasts and plasma cells were often observed in the DC cultures.

*Control Group Normal Donor Rats*

32 chambers of this group were examined and in only one an erythroid colony was observed (3 1%) No granulocytic colonies developed in the control group However in most chambers examined, macrophage colonies were observed

## Discussion

The results of the present study in rats disclose a yet undescribed phenomenon i e commitment of previously uncommitted spleen stem cells in response to a severe and abrupt depletion of replicating bone marrow cells. Studies on rats and mice have indicated that under conditions of severe hemorrhage or infections the spleen takes part in the production of red and white blood cells [9 10 13 14] In a previous investigation on rats we reported on the administration of a single intraperitoneal injection of HU which induced necrosis mainly to replicating erythroblasts [3] This rather selective cellular damage is apparently due to the fact that erythroblasts represent the majority of cells in the S phase of the cycle by the time of exposure to HU [3] Through a yet unknown stimulus, the information of reduction of replicating cells in the bone marrow is transmitted to the spleen as indicated by the results of the present study Upon this stimulation, resting stem cells become committed to various blood cell lines.

Commitment of spleen stem cells was demonstrated here by their capacity to initiate the growth of diffusion chamber hematopoietic colonies.

*Fig 5* Macrophages in DC colony The cells grow in small groups of 2 3 or individually scattered in the fibrinous clot Light micrograph × 700

*Fig 6* A typical macrophage in colony The cytoplasm contains a large variety or organelles. Note short projections of the surface membrane Electron micrograph × 7,600


10 µm

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5
10 μm
6

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spleen lymphocytes in the rat becomes committed to various blood cell lines following HU administration. Whether these particular spleen lymphocytes migrate to the bone marrow as a result of the administration of the drug remains to be demonstrated.

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It appears that the process of commitment of stem cells in the spleen of rat takes place soon after replicating marrow cells have been damaged by HU Within only 1–3 h following administration of the drug, spleen cells were collected and implanted in DC. After several days of implantation, the spleen cells gave rise to blood cell colonies. RENCRICCA *et al* [20] ruled out the possibility of erythropoietin playing a role in triggering commitment of stem cells to the erythroid line in mice administered HU

It is of interest to note that, although the host mice did not receive any specific erythropoietic stimulus apart from the irradiation erythroid colonies developed in 15 out of 56 DC. Growth of peritoneal DC erythroid colonies in the absence of a potent erythropoietic stimulus applied to the host animals is an unusual phenomenon [7] A possible explanation for this process may be related to the enrichment of stem cells for the erythroid line found in the original inoculum This degree of enrichment may be able to overcome the unfavorable growth conditions of the peritoneal cavity for this line In view of the previously reported effect of a single dose of HU damaging preferentially marrow erythroblasts [3] commitment of spleen stem cells mainly to this line may possibly account for growth of DC erythroid colonies in the absence of erythropoietic stimuli applied to the hosts.

Unlike studies of DC granulocytic and macrophage colonies initiated by implantation of bone marrow cells or of leukocytes from peripheral blood [1 17] in the present investigation of implanted spleen cells it was observed that the two lines do not grow to an equal extent In the experimental group which included 56 chambers, granulocytic colonies were observed in only 4 cultures while macrophages were noted in most of them. Moreover in the control group granulocytic colonies did not develop while macrophages were observed in all 32 chambers examined Studies of *in vitro* and of DC cultures derived from bone marrow and blood leukocytes suggest a common stem cell origin of granuloid and macrophage cells [1 8 12 19] It is possible, however that in the rat spleen there are separate stem cells for these two lines and therefore they give rise to granulocytic and macrophage colonies of various magnitude However this hypothesis needs further support.

In a previous report on rats administered a single dose of HU we concluded that following the initial killing of marrow cells in the S phase of the cycle there is an influx of lymphoid cells from an extramedullary source. This stream of lymphocytes was shown to be enriched in stem cells [3] The present investigation merely proves that a population of

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# Transient Monoclonal Immunoglobulin G with Anti-Dextran Activity[1]

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*Key Words.* Anti-dextran · Transient IgG-*K*

*Abstract.* A patient with regional enteritis had received iron dextran for treatment of iron deficiency. Subsequently he developed a large (3.1 g/100 ml) IgG-*K* serum spike which had precipitin activity against dextran sulfate but not a variety of other antigens. There has been no evidence of multiple myeloma and the spike gradually disappeared spontaneously over the course of 2 years. We speculate that the monoclonal protein may have developed as a response to the iron dextran injections under the immunologic stress of a chronic inflammatory disease.

Monoclonal immunoglobulin spikes are usually seen in multiple myeloma, progressing malignancy or in a persistent form as a benign monoclonal gammopathy. The appearance of a transient serum immunoglobulin spike is known and has been associated with a number of conditions, most often infection [6–10]. In some of these instances, the monoclonal immunoglobulin has had immunologic activity against the infectious agent [4–6]. In this report we describe the transient appearance of a monoclonal serum immunoglobulin spike whose immunologic activity appeared to be directed against dextran, in a man with regional enteritis, following treatment with iron dextran (Imferon).

1 Supported in part by grant No. CA 05634-16 awarded by the National Cancer Institute, Department Health, Education and Welfare.

2 We wish to thank Dr. Sidney Stein and NCI Immunoglobulin Reference Center for providing the precipitin screening tests.

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## *Case Study*

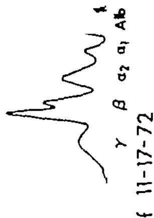
The patient was first seen t Duke in 1961 at the age of 29 with epigastric pain, melena, bloody diarrhea, and the radiologic signs of pyloric channel ulcer He continued to have gastrointestinal blood loss, and in January 1965 an ileal study showed dilatation and segmentation of the small bowel and terminal ileal narrowing, resembling the classic "string sign of regional enteritis. Proctoscopy to 22 cm revealed normal mucosa. Total protein was 8 0 g/100 ml, with albumin 2.1 g/100 ml. During this hospitalization the patient received 6 ml Imferon i.m. Because of continuing blood loss and increasing symptoms of gastric outlet obstruction, in September 1965 the patient underwent vagotomy and drainage with partial gastric resection. The surgeon noted an abnormal section of terminal ileum showing the classic features of regional enteritis

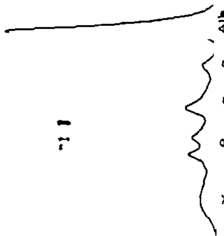
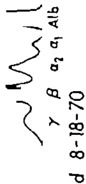
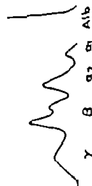
The patient continued to have intermittently guaiac positive stools, and by May 1968 his hematocrit had fallen from 44%/ 2 years earlier to 22%/ t which time he was started on course of intramuscular Imferon (total 48 ml) in divided doses. In September 1968, the patient was hospitalized with hematocrit of 26% A marrow aspirate showed the expected erythroid hyperplasia with normal maturation, but 10% plasma cells were found, including some abnormal forms. Total protein was 6.8 g/100 ml with albumin 2 0 g/100 ml. Serum electrophoresis (SEP) showed $\beta$-2 spike of 3.1 g/100 ml, which was found to be IgG-$K$ by immunoelectrophoresis. There was no Bence Jones proteinuria or hypercalcemia, and the patient denied bone pain.

Treatment for enteritis was started with ACTH and then prednisone, tapering over the ensuing 9 months, with immediate cessation of his bloody diarrhea There was gradual reduction of the monoclonal spike to 0 98 g/100 ml by August 1970 The patient was then readmitted because of rectal bleeding with hematocrit of 28%/ and was treated with ACTH, prednisone, and Imferon 40 ml A metastatic skeletal survey urine electrophoresis, antinuclear antibody assay and latex fixation test were negative A marrow aspirate showed 4% plasma cells, ith some abnormal forms persisting The patient hematocrit climbed to 42%, but in April 1971 he was again admitted following rectal bleeding episode with hematocrit of 27% SEP showed his monoclonal spike t be further reduced to 0 38 g/100 ml H was treated again with ACTH, prednisone, and Imferon 16 ml i.m. I February 1972, the patient had normal SEP and it has remained so since. No monoclonal immunoglobulin was detected by IEP

A serum specimen obtained in 1969 hen the monoclonal spike was prominent was tested by agarose Ouchterlony immunodiffusion gainst many polysaccharide and bacterial test antigens, including xylan, dextran sulfate, *B circulans*, gum ghetti, arabinogalactan, *S* *eleco* inulin, gum carrageenin, *P mirabilis*, lactobacillus and laminarin there was strong immunoprecipitin activity with dextran sulfate only This activity was found in the IgG fraction of the serum and was not detectable in serum obtained from the patient subsequent t February 1972.

*Fig 1* The initial serum electrophoresis and subsequent studies demonstrating the progressive decrease in the abnormal component and its ultimate disappearance







a 9-9-68
b 9-17-68
c 1-10-69
d 8-18-70
e 4 1-71
f 11-17-72
γ β α2 α1 Alb

of increased numbers of reactive plasma cells in his bone marrow could have responded to the administration of dextran, a polysaccharide antigen, with a monoclonal antibody in a situation analogous to that in the rabbit and human immunization experiments.

It is not clear why the monoclonal spike decreased steadily from the time it was first measured, with no anamnestic response despite two subsequent injections. The route of administration of the Imferon was not different, nor was the activity of the patient's regional enteritis, at the time of each injection. The only significant difference between the Imferon injections given prior to the discovery of the monoclonal spike and those given later is that at the time of the later injections the patient received ACTH and prednisone simultaneously with the Imferon. Thus, the administration of steroids, known to be immunosuppressive, together with the dextran preparation, might have prevented an anamnestic response to the immunogen.

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*Discussion*

Monoclonal immunoglobulin spikes are most often seen in multiple *myeloma, a malignant proliferation of plasma cells and in benign mono*clonal gammopathy It has been suggested that chronic immune stimulation might result at first in a reactive plasmacytosis then in a transition to a malignant proliferation in some cases [8] Many patients with chronic inflammatory diseases have bone marrow plasmacytosis [3] and many patients with myeloma have had antecedent inflammatory disorders [7] The findings of antibody like activity in some monoclonal immunoglobulins in these disorders [2 9] has also suggested that antigenic stimulation may play a role in the genesis of the disease However these monoclonal im munoglobulins persist or progressively increase unlike those of true im munologic reactivity Occasionally a monoclonal spike disappears, con forming more to the pattern of true immunologic reactivity [6 10] Such immunoglobulins are occasionally seen in infection and a few have been found to have antibody like activity against the infectious agent [1 4] Our patient appears to fall into this last group with a transient monoclon al immunoglobulin spike His M-spike is unusual because its highest *measured level of 3 1 g/100 ml is in excess of that seen with the usual be*nign monoclonal spike, and because it appears to have immunoprecipitin activity against dextran sulfate

The appearance of a monoclonal immunoglobulin spike as a reactive phenomenon is best documented in animals. For example, following the intraperitoneal injection of mineral oil plastics or other substances capable of behaving as chronic reticuloendothelial system stimulants, one can observe in mice the information first of a granulomatous peritonitis, fol lowed by the appearance of plasmacytomas secreting monoclonal immu noglobulin often of the IgA type [8] Many of these monoclonal immunoglobulins *have had antibody activity* One can also produce true homogenous antibodies against polysaccharide antigens in healthy laboratory rabbits with proper immunization [5] Moreover it has been shown that the antibodies produced in humans immunized with dextran and other polysaccharides have restricted heterogeneity with respect to heavy chain subtype and light chain, and genetic markers when subfractionated on the basis of specificity for glycosidic linkage or size of combining site [11]

This patient with regional enteritis, a chronic inflammatory disease with chronic reticuloendothelial stimulation supported by the appearance

of increased numbers of reactive plasma cells in his bone marrow could have responded to the administration of dextran, a polysaccharide antigen, with a monoclonal antibody in a situation analogous to that in the rabbit and human immunization experiments.

It is not clear why the monoclonal spike decreased steadily from the time it was first measured, with no anamnestic response despite two subsequent injections. The route of administration of the Imferon was not different, nor was the activity of the patient's regional enteritis, at the time of each injection. The only significant difference between the Imferon injections given prior to the discovery of the monoclonal spike and those given later is that at the time of the later injections the patient received ACTH and prednisone simultaneously with the Imferon. Thus, the administration of steroids, known to be immunosuppressive, together with the dextran preparation, might have prevented an anamnestic response to the immunogen.

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# Platelet Functions in Dysproteinaemia

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*Key Words.* Altered platelet function  Multiple myeloma  Macroglobulinaemia

*Abstract.* Platelet functions were studied in 16 patients with multiple myeloma (MM) and 4 with primary macroglobulinemia (PM). The fall in immunoglobulins on therapy was correlated with the status of platelet function. Effect of incubation of normal platelets with immunoglobulins derived from these patients on kaolin-induced platelet factor 3 (PF3) release was studied to elucidate the mechanism of platelet function defect. Results show increased bleeding time, absence or poor platelet adhesion and aggregation, poor PF3 availability and reduced total PF3 in platelets more consistently in PM but to a lesser extent in MM. Normalisation or partial reduction in globulins on therapy was associated with improvement in platelet functions. Incubation of normal platelets with immunoglobulins enhanced the kaolin-induced PF3 availability. It is suggested that *in vivo* platelet activation may bring about the PF3 release which subsequently manifests as poor PF3 availability and reduction in its total contents.

The role of altered platelet functions in dysproteinaemia has been the subject of some recent studies [1–9]. In an earlier communication from this laboratory it was reported that in macroglobulinaemia, platelet adhesion, aggregation platelet factor 3 (PF3) release and its total contents are all affected [10]. It was also suggested that these changes could be due to the presence of macroglobulins on the platelets which interfered with their function. The pathogenesis, however was not fully understood. It was also not clear whether only macroglobulins or other immunoglobulins also present in increased concentration would have similar effects on pla

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telets. The present studies, therefore, were carried out in patients of multiple myeloma (MM) with increased amounts of immunoglobulins A, G and M as well as of primary macroglobulinaemia. The effect of chemotherapy resulting in a fall in immunoglobulin was correlated with the status of platelet functions. *In vitro* incubation of normal platelets with immunoglobulins from IgA and IgG myeloma and primary macroglobulinaemia (PM) and subsequent release reaction with kaolin, was studied to investigate the possible mechanism of platelet defects due to increase in globulins. It is suggested that globulins may activate the platelets bringing about a reversible aggregation and release reaction (PF3 release) which subsequently *in vitro* manifests as poor PF3 availability and reduction in total contents.

## *Material and Methods*

20 patients (MM 16, and PM 4) were studied. The diagnosis f these was based on clinical and radiological profile, morphologic changes in bone marrow altered serum proteins, and immunoglobulins. The diagnosis of myeloma in case 9 was made on biopsy from maxillary bone Presence of symptoms of haemorrhage was not selection criteria. A regular follow-up varying from 1 to 4 years was maintained. Each of these patients was investigated for changes in serum proteins and platelet function initially and during the follow-up. Platelet count was done by formal red cell diluent [11] *I vi* platelet adhesion was performed according to the method of *Borchgrevink* [12] in those cases where bleeding time was prolonged. Platelet aggregation was done by visual method [11] after adding ADP adrenaline, noradrenaline, thrombin and collagen. It wa measured in 2 cases of PM (No. 19, 20) by chronolog aggregometer using 0.5 1.5 2.5 5 and 7.5 g/0.02 ml of ADP adrenaline and noradrenaline Detailed immunoglobulin analysis was done PF3 release and total PF3 activity of the platelets were estimated as earlier [10] and were also read on mean dilution curve obtained in 51 controls from mean calcium stypven time (CS time) of frozen and thawed platelet-rich plasma [13].

The mode of action of globulins was tested *in vitro* in the following manner Normal and patients sera were treated with saturated ammonium sulphate, the precipitate was twice washed with ammonium sulphate (33.3% ) and then dialysed with large quantities f buffered phosphate saline ($NaH_2PO$ pH 7.2) at 4 °C. The buffer was frequently changed The globulins were stored at 20 °C.

*PF3 release of normal platelets* was tested on incubation with normal and patients globulin for 30 min at 37 C in water bath. PF3 release was measured by adding kaolin and calcium stypven time was determined at 1 min and at intervals of 5 min up to 20 min. However similar pattern was noted at 10 and 20 min. Hence in later experiments, the stypven time was noted at 1 and 10 min only Additional controls were put with normal and patients globulins without prior incubation. Buffer controls were carried out simultaneously The globulins obtained from 3 cases of

IgG myeloma, one of IgA myeloma and two of PM were tested. Each globulin was tested 2–5 times with different normal platelets. Globulins obtained from single patient at different time intervals was also tested using same normal platelets.

The quantity of globulins in the majority of these preparations was between 1.7 and 2.7 g/dl However in 1 case of PM 3 and 4 g/dl of globulin were noted at two different occasions.

## *Observations and Results*

Only 1 patient with PM (No 18) presented with epistaxis at the time of initial study None of these cases had bleeding occurring as a complication during follow up

Anaemia was found in 17 patients on initial evaluation It was, however severe ($\leq 5$ g/dl) in 3 Serum albumin was markedly decreased ($< 3$ g/dl) in 15 patients (table I) In a single case, an electrophoretic spike was seen with normal immunoglobulin levels (No 9) Platelet functions studied in these patients are shown in table II Platelet counts and morphology were normal during the initial and follow up studies. Markedly poor *in vivo* platelet adhesion was noted in 6 of 8 cases studied. All 4 cases of PM and 2 cases of IgG myeloma showed poor platelet adhesion

There was complete absence of platelet aggregation in 3 cases of PM (No 17 19 20) in two of these the chronolog aggregometer did not record any aggregation either with 75 $\mu$g of ADP or with adrenaline (No 19 20) However in case 18 platelet aggregation was abnormal with ADP and adrenaline but was within 2 SDs with noradrenaline thrombin and collagen

Complete absence of platelet aggregation was observed in 1 case (No 3) of IgG myeloma. In another (No 2) it was abnormal with ADP adrenaline and thrombin but was within 2 SDs with noradrenaline. Patients of IgA myeloma did not reveal any aggregation abnormality at the time of initial study

PF3 release would depend upon the presence of normal or decreased amount of it in the platelets It was calculated from the standard graph made from 51 controls [13] In 4 cases of IgG myeloma (No 3 4 5 7) there was a reduced total amount of PF3 with poor PF3 release (table II) The release of PF3 however was of the order of 1.5 3 10.5 and 17.5%, respectively (normal 20–40%) In the latter 2 the decreased rate was proportionate to the reduced total amount of PF3 In the other 3 patients (No 1 2, 6) there was a true PF3 release defect (14 2 6 and 19%, respectively) the total quantity being normal in them (table II) 2 patients

*Table I* Serum proteins and immunoglobulin levels in dysproteinemia

| Patient | | Hb, g/dl | Total serum proteins, g/dl | Albumin, g/dl | Globulin, g/dl | Immunoglobulin, U IgG | IgA | IgM | Type of myeloma |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A. R. | 5.9 | 11 | 2.37 | 8.65 | | | – | IgG |
| 2 | K. D | 8.0 | 14 60 | 1.55 | 13 05 | | | | IgG |
| | K. D | 10.4 | 9.5 | 3.25 | 6.25 | 1070 | 12.9 | 54.2 | IgG |
| 3 | K. L. | 4.7 | 10.55 | 2.80 | 7 75 | | | | IgG |
| | K. L. | 10.8 | 6.3 | 3 7 | 2 6 | | | | IgG |
| 4 | G S. | 10.8 | 11 10 | 4.63 | 8 55 | | | | IgG |
| | G S. | 10.9 | 11 45 | 2.10 | 9.35 | 1030 | 8.4 | 297 | IgG |
| 5 | R. L. | 11.0 | 7.00 | 3.45 | 3 65 | | | | IgG |
| 6 | P C. | 13.3 | 7.60 | 3.80 | 3 80 | 320 | 19 | 115 | IgG |
| 7 | A. M. | 9 8 | 11 45 | 2.4 | 9 05 | 480 | 10 | 175 | IgG |
| 8 | K. K. | 14.8 | 9.5 | 2 95 | 6.55 | | | – | IgG |
| 9 | R. A. | 11.3 | 7 60 | 3 15 | 4 45 | 126 | 192 | 140 | IgG no specific change in immuno-globulin |
| 10 | O. R. | 8.0 | 9.5 | 2.2 | 7.3 | | | | IgA |
| 11 | S. N. | 8.1 | 9 65 | 2.10 | 7.55 | 344 | 345 | 26 2 | IgA |
| | S. N | 8.5 | 10.25 | 2.65 | 7 60 | | | | IgA |
| 12 | R. K. | 13.2 | 6.3 | 3 15 | 3 15 | 59 | 96.8 | 26 2 | IgA |
| | R. K. | 13.2 | 8 35 | 3.25 | 5 10 | | | | IgA |
| 13 | P S. | 10 7 | 9.5 | 4.6 | 6.9 | 79 | 104 | 35 | IgA |
| | P S. | 10 0 | 11 4 | 2.8 | 8 6 | | | | IgA |
| 14 | J R. | 9.2 | 10 9 | 2.0 | 8.9 | 113 | 1450 | 230 | IgA |
| | J R. | 10 8 | 6 6 | 4 25 | 2.35 | 309 | 726 | 168 | IgA |
| 15 | K. C. | 8 2 | 6.5 | 3 4 | 3.1 | 112 4 | 14 5 | 17.5 | Bence-Jones myeloma |
| 16 | S. B. | 9 0 | 8.6 | 2.4 | 6 2 | 387 | 30 2 | 872 | IgM |
| 17 | S. K. | 6.7 | 10.55 | 1 70 | 8.85 | | | | primary macro-globulin-emia |
| | S K. | 9.8 | 8.65 | 1.9 | 6 75 | | | | PM |
| 18 | M. R. | 5 0 | 10.25 | 2.40 | 7 85 | | | | PM |
| | M. R. | 10 5 | 7.2 | 3.3 | 3.9 | 21.0 | 8 4 | 390 | PM |
| 19 | S. T | 5 9 | 9.9 | 1 6 | 8 3 | | | very high | PM |
| 20 | T C. | 4.9 | 10 1 | 1.25 | 8 85 | 256 | 33.4 | 990 5 | PM |

Follow-up.

IgG myeloma, one of IgA myeloma and two of PM were tested. Each globulin was tested ~5 times with different normal platelets. Globulins obtained from single patient at different time intervals was also tested using same normal platelets.

The quantity of globulins in the majority of these preparations was between 1.7 and 2.7 g/dl. However in 1 case of PM 3 and 4 g/dl of globulin were noted at two different occasions.

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| Patient | | Hb, g/dl | Total serum proteins, g/dl | Albumin, g/dl | Globulin, g/dl | Immunoglobulin, U | | | Type of myeloma |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | IgG | IgA | IgM | |
| 1 | A.R. | 5.9 | 11 | [illegible]37 | 8.65 | | | | IgG |
| 2 | K.D | 8.0 | 14.60 | 1.55 | 13.05 | | | | IgG |
| | K.D | 10.4 | 9.5 | 3.[illegible]5 | 6.25 | 1070 | 1[illegible]9 | 54.2 | IgG |
| 3 | K.L. | 4.7 | 10.55 | [illegible]80 | 7.75 | | | | IgG |
| | K.L. | 10.8 | 6.3 | 3.7 | [illegible]6 | | | | IgG |
| 4 | G.S | 10.8 | 11.10 | [illegible]65 | 8.55 | | | | IgG |
| | G.S. | 10.9 | 11.45 | [illegible]10 | 9.35 | 1030 | 8.4 | [illegible]97 | IgG |
| 5 | R.L. | 11.0 | 7.00 | 3.45 | 3.65 | | | | IgG |
| 6 | P.C. | 13.3 | 7.60 | 3.80 | 3.80 | 320 | 19 | 115 | IgG |
| 7 | A.M | 9.8 | 11.45 | [illegible]4 | 9.05 | 480 | 10 | 175 | IgG |
| 8 | K.K. | 14.8 | 9.5 | [illegible]95 | 6.55 | | | | IgG |
| 9 | R.A. | 11.3 | 7.60 | 3.15 | 4.45 | 126 | 192 | 140 | IgG no specific change in immuno-globulin |
| 10 | G.R. | 8.0 | 9.5 | [illegible] | 7.3 | | | | IgA |
| 11 | S.N. | 8.1 | 9.65 | [illegible]10 | 7.55 | 344 | 345 | 26.2 | IgA |
| | S.N. | 8.5 | 10.25 | [illegible]65 | 7.60 | | | | IgA |
| 12 | R.K. | 13.[illegible] | 6.3 | 3.15 | 3.15 | 59 | 96.8 | 26.2 | IgA |
| | R.K. | 13.2 | 8.35 | 3.[illegible]5 | 5.10 | | | | IgA |
| 13 | P.S. | 10.7 | 9.5 | 2.6 | 6.9 | 79 | 104 | 35 | IgA |
| | P.S | 10.0 | 11.4 | [illegible]8 | 8.6 | | | | IgA |
| 14 | J.R. | 9.2 | 10.9 | 2.0 | 8.9 | 113 | 1450 | [illegible]30 | IgA |
| | J.R. | 10.8 | 6.6 | 4.25 | 2.35 | 309 | 726 | 168 | IgA |
| 15 | K.C | 8.2 | 6.5 | 3.4 | 3.1 | 112.4 | 14.5 | 17.5 | Bence-Jones myeloma |
| 16 | S.B | 9.0 | 8.6 | [illegible]4 | 6.2 | 387 | 30.2 | 87[illegible] | IgM |
| 17 | S.K. | 6.7 | 10.55 | 1.70 | 8.85 | | | | primary macro-globulin-emia |
| | S.K. | 9.8 | 8.65 | 1.9 | 6.75 | | | | PM |
| 18 | M.R. | 5.0 | 10.25 | 2.40 | 7.85 | | | | PM |
| | M.R. | 10.5 | 7.2 | 3.3 | 3.9 | 21.0 | 8.4 | 390 | PM |
| 19 | S.T | 5.9 | 9.9 | 1.6 | 8.3 | | | very high | PM |
| 20 | T.C. | 4.9 | 10.1 | 1.25 | 8.85 | 256 | 33.6 | 990.5 | PM |

Follow-up

IgG myeloma, one of IgA myeloma and two of PM were tested. Each globulin was tested 2–5 times with different normal platelets. Globulins obtained from single patient at different time intervals was also tested using same normal platelets.

The quantity of globulins in the majority of these preparations was between 17 and ~7 g/dl. However in 1 case of PM 3 and 4 g/dl of globulin were noted at two different occasions.

*Observations and Results*

Only 1 patient with PM (No 18) presented with epistaxis at the time of initial study None of these cases had bleeding occurring as a complication during follow up

Anaemia was found in 17 patients on initial evaluation It was, however severe ($\leq 5$ g/dl) in 3 Serum albumin was markedly decreased ($<3$ g/dl) in 15 patients (table I) In a single case, an electrophoretic spike was seen with normal immunoglobulin levels (No 9) Platelet functions studied in these patients are shown in table II Platelet counts and morphology were normal during the initial and follow up studies. Markedly poor *in vivo* platelet adhesion was noted in 6 of 8 cases studied. All 4 cases of PM and 2 cases of IgG myeloma showed poor platelet adhesion

There was complete absence of platelet aggregation in 3 cases of PM (No 17 19 20) in two of these the chronolog aggregometer did not record any aggregation either with 75 $\mu$g of ADP or with adrenaline (No 19 20) However in case 18, platelet aggregation was abnormal with ADP and adrenaline, but was within 2 SDs with noradrenaline thrombin and collagen

Complete absence of platelet aggregation was observed in 1 case (No 3) of IgG myeloma In another (No 2) it was abnormal with ADP adrenaline and thrombin but was within 2 SDs with noradrenaline. Patients of IgA myeloma did not reveal any aggregation abnormality at the time of initial study

PF3 release would depend upon the presence of normal or decreased amount of it in the platelets It was calculated from the standard graph made from 51 controls [13] In 4 cases of IgG myeloma (No 3 4 5 7) there was a reduced total amount of PF3 with poor PF3 release (table II) The release of PF3 however was of the order of 1 5 3 10.5 and 17 5%, respectively (normal 20–40%) In the latter 2 the decreased rate was proportionate to the reduced total amount of PF3 In the other 3 patients (No 1 2, 6) there was a true PF3 release defect (14 2 6 and 19%, respectively) the total quantity being normal in them (table II) 2 patients

*Table III* Platelet function tests in dysproteinemia on follow-up

| Patient | | Platelet adhesion, | Platelet aggregation ADP | adrenaline | noradrenaline | thrombin | collagen | PF3 release at 20 min RVV time in sec | PF3 assay of control |
|---|---|---|---|---|---|---|---|---|---|
| | K. D | | 15 | 1 | 37 | 35 | [illegible] | 17 | 114 |
| 3 | K. L. | | 15 | 17 | [illegible] | 30 | | [illegible] | 100 |
| 4 | G S | 16.6 | 17 | 31 | 30 | 30 | 32 | 22.5 | 93 |
| 11 | S. N. | | 16 | 19 | 23 | 20 | 18 | 18.5 | 94 |
| 1 | R. K. | | 1 | 16 | [illegible] | 16 | 18 | 1 | 105 |
| 13 | P S | | 17 | 27 | 35 | [illegible] | 4 5 | 25 | 64 |
| 14 | J R. | | 13 | 18 | 20 | 17 | 18 | 4 | 94 |
| 17 | S. K. | 27_ | no aggregation | | | | | 30 5 | 43 |
| 18 | M R. | 4_ | 10 | 13 | 16 | 15 | 15 | 14 | 115 |

changed. Platelet functions and changes on follow-up are shown in tables III and IV Platelet adhesion became normal in both patients of PM (No. 17 18) Platelet aggregation, PF3 release and total amount of PF3 became normal in 1 (No 18), but these remained abnormal in another (No. 17).

In IgG myeloma, in 1 patient, abnormalities of platelet aggregation and adhesion appeared and poor PF3 release persisted (No. 4). In another patient (No 2) PF3 release became normal but platelet aggregation was still abnormal Partial improvement in platelet aggregation and PF3 release, however was noted in case 3 with normalization of PF3 contents.

Mild abnormality of platelet aggregation was seen for the first time in 1 patient of IgA myeloma (No. 13), in whom poor PF3 release persisted with further reduction of total PF3 contents. True poor PF3 release defect was still seen in case 14 (with normal PF3 contents) and freshly appeared in case 12.

### *Effect of Globulin With and Without Incubation on Platelet Function*

Results of incubation of normal platelets with globulin and subsequent PF3 availability are shown in table V Stypven time of PRP at 10 min in cases of normal and patient s globulins reveal that PF3 availability is enhanced, as compared to control with buffer Significant differences are seen between the groups 1 and 3 ($p < 0.01$) and 2 and 3 ($p < 0.01$). No signifi-

*Table II* Platelet adhesion, aggregation, PF3 release and assay in 20 patients

| Patient | Platelet adhesion | Platelet aggregation sec ADP | adre-naline | noradre-naline | thrombin | collagen | PF3 release at 20 min RVV time in sec | PF3 assay of control |
|---|---|---|---|---|---|---|---|---|
| 1 | 7 8 | 12 | 15 | 20 | 19 | – | 1 5 | 104 |
| | | 15 | 25 | 7 | 29 | – | 29.5 | 94 |
| 3 | – | no aggregation | | | | | 31.5 | 45 |
| 4 | 7 00 | 12 | 18 | 18 | 6 | – | 28.0 | 80 |
| 5 | 0 95 | 12 | 17 | 1 | 17 | 19 | 23 | 54 |
| 6 | | 14 | 19 | 2_ | 15 | 16 | 20 | 104 |
| 7 | – | 14 | 17 | 18 | 15 | 15 | 20.5 | 69 |
| 8 | – | 13 | 16 | 18 | 17 | 17 | 18 | 100 |
| 9 | | 14 | 16 | 21 | 17 | 16 | 20 5 | 104 |
| 10 | | 14 | 18 | 21 | 18 | 19 | 25.5 | 94 |
| 11 | 33 5 | 1 | 17 | 22 | 18 | 18 | 17 | 104 |
| 1 | – | 15 | 18 | 2_ | 17 | 18 | 16 | 83 |
| 13 | – | 1 | 18 | 20 | 17 | 17 | 20.5 | 72 |
| 14 | – | 14 | 18 | 2_ | 18 | 17 | 24.5 | 100 |
| 15 | – | 1 | 18 | — | 18 | 19 | 15.5 | 112 |
| 16 | | 1 | 17 | 19 | 17 | 20 | 17 5 | 83 |
| 17 | 14 7 | no aggregation | | | | | 36.5 | 25 |
| 18 | 7 05 | 1 | 4 | 27 | 23 | 19 | 30.5 | 40 |
| 19 | 1_0 | no aggregation | | | | | 37.5 | 14 |
| 20 | 15 1 | no aggregation | | | | | 31 | 18 |
| Normal values | 20 | 11_ ± 1 6 | 16.7 ± 1 6 | 20.3 ± 3 6 | 19 1 ± 3 9 | 17 6 ± 1 8 | 17 3 ± 1.23 | 90 112 |

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with IgA myeloma (No 10 14) had true PF3 release defect (5 4 and 7%, respectively) with normal total PF3 in them In the third case (No 13), the PF3 release was proportionate to the total amount present (17.5% release with total PF3 amount being 72%)

In all 4 patients of PM severe reduction in total PF3 contents with true PF3 release defects were seen PF3 release was not proportionate to the total amount present the release was of the order of 1–1.5 % in all of them

Follow up studies shown in table I indicate partial reduction of serum globulins in 4 (No 3 14 17 18) and increased globulin levels in 3 (No 4 12, 13) patients. In 1 (No 11) globulin level remained un

*Table III* Platelet function tests in dysproteinemia on follow-up

| Patient | | Platelet adhesion, % | Platelet aggregation | | | | | PF3 release (20 min RVV time in sec) | PF3 assay % of control |
|---|---|---|---|---|---|---|---|---|---|
| | | | ADP | adrenaline | noradrenaline | thrombin | collagen | | |
| | K. D | | 15 | 1 | 37 | 35 | [illegible] | 17 | 114 |
| 3 | K. L. | | 15 | 17 | [illegible]7 | 30 | | [illegible]3 | 100 |
| 4 | G. S. | 16.6 | 17 | 31 | 30 | 30 | [illegible] | [illegible]5 | 93 |
| 11 | S. N. | | 16 | 19 | [illegible]3 | 20 | 18 | 18.5 | 94 |
| 1 | R. K. | | 1 | 16 | 20 | 16 | 18 | 21 | 105 |
| 13 | P. S. | | 17 | [illegible]7 | 35 | 25 | 4.5 | 25 | 64 |
| 14 | J. R. | | 13 | 18 | [illegible]0 | 17 | 18 | 4 | 94 |
| 17 | S. K. | [illegible] | no aggregation | | | | | 30.5 | 43 |
| 18 | M. R. | 4.2 | 10 | 13 | 16 | 15 | 15 | 14 | 115 |

changed. Platelet functions and changes on follow-up are shown in tables III and IV Platelet adhesion became normal in both patients of PM (No 17 18). Platelet aggregation, PF3 release and total amount of PF3 became normal in 1 (No. 18), but these remained abnormal in another (No. 17).

In IgG myeloma, in 1 patient, abnormalities of platelet aggregation and adhesion appeared and poor PF3 release persisted (No. 4) In another patient (No 2) PF3 release became normal but platelet aggregation was still abnormal. Partial improvement in platelet aggregation and PF3 release, however was noted in case 3 with normalization of PF3 contents.

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Results of incubation of normal platelets with globulin and subsequent PF3 availability are shown in table V Stypven time of PRP at 10 min in cases of normal and patient s globulins reveal that PF3 availability is enhanced, as compared to control with buffer Significant differences are seen between the groups 1 and 3 ($p < 0.01$) and 2 and 3 ($p < 0.01$) No signifi-

*Table II* Platelet adhesion, aggregation PF3 release and assay in 20 patients

| Patient | Platelet adhesion | Platelet aggregation sec ADP | adrenaline | noradrenaline | thrombin | collagen | PF3 release at 30 min RVV time in sec | PF3 assay % of control |
|---|---|---|---|---|---|---|---|---|
| 1 | 7 8 | 1 | 15 | 20 | 19 | – | 1.5 | 104 |
| 2 | – | 15 | 25 | 27 | 29 | – | 29.5 | 94 |
| 3 | | no aggregation | | | | | 31.5 | 45 |
| 4 | 27 00 | 1 | 18 | 18 | 26 | – | 28 0 | 80 |
| 5 | 0 95 | 1 | 17 | 21 | 17 | 19 | [illegible]3 | 54 |
| 6 | | 14 | 19 | 2[illegible] | 15 | 16 | 20 | 104 |
| 7 | | 14 | 17 | 18 | 15 | 15 | 20.5 | 69 |
| 8 | – | 13 | 16 | 18 | 17 | 17 | 18 | 100 |
| 9 | | 14 | 16 | 1 | 17 | 16 | 30.5 | 104 |
| 10 | – | 14 | 18 | 21 | 18 | 19 | 25.5 | 94 |
| 11 | 33.5 | 1 | 17 | 2[illegible] | 18 | 18 | 17 | 104 |
| 1[illegible] | | 15 | 18 | [illegible] | 17 | 18 | 16 | 83 |
| 13 | – | 1 | 18 | 20 | 17 | 17 | 30.5 | 77 |
| 14 | – | 14 | 18 | 22 | 18 | 17 | 4.5 | 100 |
| 15 | – | 1 | 18 | [illegible]2 | 18 | 19 | 15.5 | 11 |
| 16 | | 1 | 17 | 19 | 17 | [illegible]0 | 17.5 | 83 |
| 17 | 14 7 | no aggregation | | | | | 36.5 | [illegible]5 |
| 18 | 7 05 | 21 | 4 | [illegible]7 | [illegible]3 | 19 | 30.5 | 40 |
| 19 | 1[illegible]0 | no aggregation | | | | | 37.5 | 14 |
| 20 | 15 1 | no aggregation | | | | | 31 | 18 |
| Normal values | >20 | 11 2± 1 6 | 16.7± 1 6 | 20.3± 3 6 | 19 1± 3 9 | 17 6± 1 8 | 17.3± 1.[illegible]3 | 90-11[illegible] |

– = Not done.

with IgA myeloma (No 10 14) had true PF3 release defect (5 4 and 7%, respectively) with normal total PF3 in them. In the third case (No 13) the PF3 release was proportionate to the total amount present (17.5% release with total PF3 amount being 72%)

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|---|---|---|---|---|---|---|---|---|
| | | ADP | adrenaline | noradrenaline | thrombin | collagen | | |
| K. D | | 15 | 1 | 37 | 35 | 23 | 17 | 114 |
| 3 K. L. | | 15 | 17 | 27 | 30 | | 23 | 100 |
| 4 O S | 16 6 | 17 | 31 | 30 | 30 | 32 | 22.5 | 93 |
| 11 S N | | 16 | 19 | [illegible]3 | 20 | 18 | 18.5 | 94 |
| 1 R. K. | | 1 | 16 | 20 | 16 | 18 | 1 | 105 |
| 13 P S | | 17 | [illegible]7 | 35 | [illegible]5 | 4 5 | [illegible]5 | 64 |
| 14 J R. | | 13 | 18 | 20 | 17 | 18 | 4 | 94 |
| 17 S. K. | 27[illegible] | no aggregation | | | | | 30 5 | 43 |
| 18 M. R. | 4[illegible] | 10 | 13 | 16 | 15 | 15 | 14 | 115 |

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| 1 | 7 8 | 1 | 15 | 20 | 19 | – | 21.5 | 104 |
| | – | 15 | 25 | 27 | 29 | – | 29 5 | 94 |
| 3 | – | no aggregation | | | | | 31.5 | 45 |
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| 5 | 0 95 | 1 | 17 | 21 | 17 | 19 | 23 | 54 |
| 6 | – | 14 | 19 | – | 15 | 16 | 20 | 104 |
| 7 | – | 14 | 17 | 18 | 15 | 15 | 20.5 | 69 |
| 8 | – | 13 | 16 | 18 | 17 | 17 | 18 | 100 |
| 9 | – | 14 | 16 | 21 | 17 | 16 | 20.5 | 104 |
| 10 | – | 14 | 18 | 21 | 18 | 19 | 25.5 | 94 |
| 11 | 33.5 | 12 | 17 | 22 | 18 | 18 | 17 | 104 |
| 12 | – | 15 | 18 | 22 | 17 | 18 | 16 | 83 |
| 13 | – | 1 | 18 | 20 | 17 | 17 | 20 5 | 72 |
| 14 | – | 14 | 18 | 2. | 18 | 17 | 4 5 | 100 |
| 15 | – | 1 | 18 | 2. | 18 | 19 | 15.5 | 112 |
| 16 | | 1 | 17 | 19 | 17 | 20 | 17.5 | 83 |
| 17 | 14.7 | no aggregation | | | | | 36.5 | 25 |
| 18 | 7 05 | 21 | 4 | 7 | .3 | 19 | 30.5 | 40 |
| 19 | 12.0 | no aggregation | | | | | 37.5 | 14 |
| 20 | 15 1 | no aggregation | | | | | 31 | 18 |
| Normal values | >20 | 11 ± 1 6 | 16 7± 1 6 | 20.3± 3 6 | 19 1± 3.9 | 17 6± 1 8 | 17 3± 1.23 | 90 112 |

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with IgA myeloma (No 10 14) had true PF3 release defect (5 4 and 7%, respectively) with normal total PF3 in them In the third case (No 13), the PF3 release was proportionate to the total amount present (17.5% release with total PF3 amount being 72%)

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|---|---|---|---|---|---|---|---|---|
| 1 | 7 8 | 1 | 15 | 20 | 19 | – | 1.5 | 104 |
| | – | 15 | 25 | 27 | 29 | – | 29.5 | 94 |
| 3 | – | no aggregation | | | | | 31.5 | 45 |
| 4 | 7 00 | 1 | 18 | 18 | 6 | – | [illegible] | 80 |
| 5 | 0.95 | 12 | 17 | 21 | 17 | 19 | [illegible] | 54 |
| 6 | – | 14 | 19 | 22 | 15 | 16 | 20 | 104 |
| 7 | – | 14 | 17 | 18 | 15 | 15 | 20.5 | 69 |
| 8 | – | 13 | 16 | 18 | 17 | 17 | 18 | 100 |
| 9 | – | 14 | 16 | 21 | 17 | 16 | 20.5 | 104 |
| 10 | – | 14 | 18 | 1 | 18 | 19 | 25.5 | 94 |
| 11 | 33 5 | 1 | 17 | [illegible] | 18 | 18 | 17 | 104 |
| 12 | – | 15 | 18 | [illegible] | 17 | 18 | 16 | 83 |
| 13 | | 1 | 18 | 20 | 17 | 17 | 20.5 | 72 |
| 14 | – | 14 | 18 | [illegible] | 18 | 17 | 24.5 | 100 |
| 15 | – | 12 | 18 | [illegible] | 18 | 19 | 15.5 | 112 |
| 16 | | 1 | 17 | 19 | 17 | 20 | 17 5 | 83 |
| 17 | 14 7 | no aggregation | | | | | 36.5 | 25 |
| 18 | 7 05 | 1 | 4 | 27 | [illegible] | 19 | 30.5 | 40 |
| 19 | [illegible] | no aggregation | | | | | 37 5 | 14 |
| 20 | 15 1 | no aggregation | | | | | 31 | 18 |
| Normal values | >20 | 11 2± 1 6 | 16 7± 1 6 | 20.3± 3 6 | 19 1± 3 9 | 17 6± 1 8 | 17 3± 1.23 | 90–112 |

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cant difference in the PF3 availability however is seen between groups 1 and 2. The incubation of platelets for 30 min at 37 C prior to addition of kaolin resulted in relatively poor PF3 release as compared to non-incubated platelets. However the release reaction did not show any obvious difference between buffer normal and patient s (groups 4 5 and 6) globulins in the latter set of experiments. This would suggest that the presence of globulin did not interfere either with the release reaction or with PF3 estimation. However incubation of platelets altered their reactivity

*Discussion*

Abnormalities of platelet function seen here are increased bleeding time absence of or poor platelet adhesion and aggregation, poor PF3 availability and reduced total PF3 contents in the platelets. All these defects are consistently seen in all the cases of macroglobulinaemia. PF3 availability is most consistently affected in IgG and to a lesser extent in IgA myeloma. The bleeding time abnormalities have been recognized earlier [1 3 4 6–8, 14] PACHTER *et al* [2] were the first to find decreased PF3 availability in patients with macroglobulinaemia, which was later confirmed by PENNY *et al* [9] Platelet adhesion and aggregation abnormalities have also been described [3 5 8]

A close correlation between platelet function and protein concentration existed in IgG and IgA myeloma. Higher immunoglobulin levels were associated with markedly altered platelet function Similar observations have been made earlier

The PF3 release was facilitated by incubation with the control and patient s globulins. However the mere presence of globulins did not have any effect. It appears that incubation retards the PF3 release, but globulins render the platelets more sensitive to it. On the basis of these *in vitro* studies, a suggestion can be made that increase in globulins reacted with the platelets and brought about the release reaction. This would result in a greater availability of PF3 for participation in the haemostatic mechanism and would also lead to reduced contents of PF3 in platelets, assuming that the releasable PF3 activity has been metabolised.

Reason for absence of platelet aggregation noted in our patients could not be accounted for in our studies. It is possible that globulins induced a reversible aggregation of the platelets *in vivo* Subsequent aggregation with various aggregating agents would then be absent *i vitro* Our at

*Table II* Changes in platelet function in MM and PM on follow-up

| Patient | Immunoglobulin studies | Platelet adhesion | Platelet aggregation | PF3 availability | PF3 assay |
|---|---|---|---|---|---|
| 1 | *IgG myeloma* | | | | |
| | 2 partial reduction | – | still poor | normal | unchanged (normal) |
| | 3 normalisation | – | improved but still poor | improved but still poor | normal |
| | 4 increased | poor | poor | remained poor | slight improvement |
| | *IgA myeloma* | | | | |
| | 11 unchanged | – | poor with ADP | unchanged | unchanged |
| | 1 increased | – | unchanged (normal) | poor | improved |
| | 13 increased | – | poor | poorer | further reduction |
| | 14 normalisation | – | unchanged (normal) | unchanged (poor) | unchanged (normal) |
| 3 | *PM* | | | | |
| | 17 partial reduction | normal | unchanged (absent) | improved but still poor | improved but still poor |
| | 18 normalisation | normal | normal | normal | normal |

*Table I* PF3 availability on incubation with globulin

| | Number of observations | Stypven time (sec) after 1 min incubation | | Stypven time (sec) after 10 min incubation | |
|---|---|---|---|---|---|
| | | range | mean | range | mean |
| IgG | 7 | 29 –40 | 34 1 | 18 28.5 | 4 4 |
| IgA | 5 | 32 –38 | 34 7 | 20 29 | 23 6 |
| P M | 12 | 1.5–47 | 34 7 | 13 31 | 23.1 |
| Combined (group 1) | 24 | 21 5–47 | 34 5 | 13 –31 | 23.7 |
| Normal globulin (group 2) | 1 | [illegible]2 –40.5 | 3[illegible]6 | 20 [illegible]8.5 | [illegible]3.5 |
| Control buffer (group 3) | 8 | 23.5–45 | 34 1 | [illegible]5–44 | 31 6 |
| Normal globulin (group 4) | 9 | 1 5–38.5 | 30.6 | 15.5–25 | 20 4 |
| Patient s globulin (group 5) | 5 | 22 39 | 30.2 | 16.0– 4.5 | 19.8 |
| Buffer (group 6) | 4 | 24 7 33 5 | 30.9 | 18 –27 5 | 20.9 |

Statistical analysis 1 and 3 $t = 3\ 07$ $p < 0.01$ 2 and 3 $t = 3.22$, $p < 0.01$

[1] PF3 availability with no prior ncubation.

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tempt to study the *in vitro* platelet aggregation of normal platelets on incubation with globulins was not successful PENNY *et al.* [9] have shown that very high concentration of globulins inhibited ADP aggregation in normal PRP in a single patient.

Normal platelet counts with extensive platelet defects without manifest symptoms of bleeding have been noted in our cases. Similar platelet function defects namely inaggregability of platelets, with or without PF3 deficiency are associated with severe bleeding from skin and mucous membrane in congenital platelet defect – thrombasthenia [16] It, therefore, appears that the defects in dysproteinaemia with regard to platelet function may not be indicative of failure of haemostatic mechanism.

The pathogenesis of bleeding in dysproteinaemias could be attributed to various mechanisms Presence of inhibitors of coagulation or non-specific reduction in clotting factors due to complexing with paraproteins [15] impaired platelet function hyperviscosity syndrome either alone or in combination can cause haemorrhagic manifestations It is possible that an *in vivo* activation of platelets by globulin releases the thromboplastic activity which may compensate for the defects in coagulation factors. However with accompanied thrombocytopenia, this compensation may fall short to counterbalance the effects of globulins inhibiting the activity of clotting factors.

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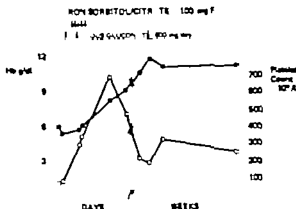



*Fig 1* Response to iron therapy

The haematological findings at presentation were as follows haemoglobin 5.6 g/dl, PCV 0.19 red cell count 3.26 10¹² l MCV 59 fl MCH 17 pg MCHC 29 g dl, white cell count 7.1 10⁹ l with normal differential count; platelets 77 10⁹ l reticulocytes 2.2% (71.7 10⁹ l). The red blood cell morphology was predominantly microcytic and hypochromic with marked aniso-poikilocytosis. Haemoglobin electrophoresis was normal and haemoglobin $A_2$ level was 1.1 %, haemoglobin F levels were not estimated. The serum iron was 13 μmol l and the total iron binding capacity 86 mol/l, the serum vitamin $B_{12}$ level was 270 ng/l and the serum and red cell folate levels were 6.2 and 353 g/l Sternal bone marrow aspiration showed normal numbers of megakaryocytes, normoblastic erythropoiesis, normal granulopoiesis and absent iron stores.

An initial diagnosis of iron deficiency anaemia with possible underlying thalassaemia was made and therapy with intramuscular iron-sorbitol/citrate complex followed by oral ferrous gluconate was started The thrombocytopenia was quickly corrected and transient thrombocytosis developed (fig 1), and the anaemia improved steadily On 30.1.75 an operative incision of the hymen was performed but since her menstrual periods were less heavy dilatation and curettage was not performed. The splenomegaly had persisted and on 4.2.75 the haematological findings showed haemoglobin 11.3 g dl PCV 0.36 RBC 5.32 × 10¹² l MCV 68 fl, MCH 21 pg; MCHC 32 g/dl reticulocytes 1.0% (56.4 × 10⁹/l) white cell and platelet counts normal haemoglobin $A_2$ 2.0%, haemoglobin F 0.4% and the presence of occasional haemoglobin H bodies The erythrocyte morphology showed mild aniso-poikilocytosis, some microcytes and occasional target cells These findings are consistent with $\alpha_1$-thalassaemia trait

## *Discussion*

The platelet count may be reduced, normal or increased in haemorrhage and other iron deficiency states [1 4 5 8, 9 12] It has also been

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# Thrombocytopenia and Iron Deficiency Anaemia in a Patient with $a_1$-Thalassaemia Trait. Response to Iron Therapy

Case Report

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*Key Words* Thrombocytopenia Iron deficiency anaemia a Thalassaemia trait

*Abstract* Iron deficiency anaemia secondary to menorrhagia was observed in a woman of Greek Cypriot origin. Moderate thrombocytopenia was also present. Treatment with parenteral and oral iron produced a transient thrombocytosis, the platelet count then returning to normal. Subsequent analysis revealed that she also carried the a thalassaemia trait. Previous reports of thrombocytopenia responsive to iron treatment are reviewed.

Thrombocytosis has long been accepted to be associated with haemorrhage [9] or iron deficiency anaemia [12] Some more recent reports have emphasised that thrombocytopenia may also be associated with iron deficiency anaemia and that the platelet count may rise in response to treatment with iron We have seen an additional patient in this category with some further interesting features.

## *Case Report*

Mrs. S. P 7-year-old woman of Greek Cypriot origin presented at St Bartholomew s Hospital in July 1974 complaining of intermittent but heavy vaginal bleeding for 9 months. She had married 9 months previously but the marriage had not been consummated. Dilatation and curettage of the uterus and incision of the hymen was planned but a routine preoperative blood count showed a haemoglobin level of 5 6 g/dl and the operation was postponed. No other cause for iron deficiency was found and the patient only complained of slight symptoms attributable to the severe anaemia. On examination, the only abnormality apart from clinical anaemia was a palpable spleen felt 2 cm below the costal margin.

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shown that reduced or even normal platelet counts show striking increases when the iron deficiency is corrected by oral or parenteral iron (cases analysed in table I).

The role of iron containing proteins (transferrin and hemin) in both protein synthesis in platelets and possibly in megakaryocytes has been demonstrated by FREEDMAN and KARPATKIN [3]. They propose that severe iron deficiency anaemia can lead to thrombocytopenia through exhaustion of an essential component compartment of iron containing protein needed for platelet synthesis [6]. However DINÇOL and AKSOY [2] have stated that in iron deficiency anaemia, platelets are at normal levels unless the situation is complicated by some other factor, for example, hypersplenism causing thrombocytopenia or active blood loss causing thrombocytosis. Their own cases [1] support this proposal and it is interesting to note the relatively great proportion of the other cases reported in which splenomegaly was demonstrated (table I). Although the description hypersplenism implies that the mechanism producing thrombocytopenia is increased pooling or destruction of platelets in the spleen, other explanations are possible. TARNUZI and SMILEY [14] suggested that the spleen produces a humoral substance which reduced the production or delivery of platelets from the bone marrow. They showed that reimplation of spleen within diffusion chambers could prevent the usual post-splenectomy thrombocytosis and postulated a humoral factor produced in the spleen which suppressed the platelet rise.

It is not clear how the possible factors in producing thrombocytopenia have acted together in our patient. There was no obvious evidence of increased splenic destruction of erythrocytes, neutrophils or platelets either at presentation or after treatment. The platelet count and haemoglobin clearly responded promptly to treatment with iron and residual effects after therapy attributable to -thalassaemia trait were characteristically mild. It seems possible that in our case, as in some of the other cases, thrombocytopenia responding to iron therapy was related in some way to splenomegaly but that it was unlikely to be mediated either by increased pooling or destruction of circulating cells.

## *Acknowledgements*

*We are grateful to Mr. D. B. FRASER for permission to publish details of this patient, and to Miss P. HARRISON for secretarial assistance.*

*Table I* Comparison of cases of thrombocytopenia responsive to iron

| Reference No | Age, years | Sex | Diagnosis | Haemoglobin g/dl | MCV fl | Platelets, $10^9$/l | Serum µg % | Bone marrow iron stores | Bone marrow mega-karyocytes | splenomegaly |
|---|---|---|---|---|---|---|---|---|---|---|
| 4 | 1 /12 | | severe infections excluded other wise not stated | 4 0 | – | 120 | 30/410 | reduced | normal | n.s. |
| 7 | 2 | F | upper respiratory tract infection | 2.1 | 52 | 42.5 | n.s. | reduced | normal | n.s. |
| | 3 | M | pneumonia (premature baby) | 3.2 | 65 | 100 | n.s. | reduced | 'adequate | 2 cm |
| | 1 1/2 | F | fever cough (premature baby) | 3.8 | 61 | 197 | n.s. | reduced | normal | no |
| | 2 4/12 | M | rhinorrhea (negro) | 4 0 | 54 | 50 | n.s. | not examined at presentation | | n.s. |
| | /12 | M | vomiting (premature baby) | 1 75 | 53 | 38 4 | n.s. | reduced | reduced | 2 cm |
| 1 | 24 | | hypersplenism[a] | 4 7 | 70 | 72 | 52/n.s. | n.s. | n.s. | yes |
| | | F | malabsorption | 5.6 | 71 | 145 | 46/n.s. | n.s. | n.s. | n.s. |
| | | F | malabsorption and chronic blood loss | 6.6 | 79 | 137 | 63/n.s. | n.s. | n.s. | n.s. |
| | | M | malabsorption | 6.5 | 77 | 121 | 36/n.s. | n.s. | n.s. | n.s. |
| 13 | 31 | F | negro, staghorn calculus, haematuria, menorrhagia | 5.2 | 66 | 40 | 34/341 | not examined | | n.s. |
| 10 | middle aged' | F | cirrhosis ascites 'hypersplenism | 7 1 | 98 | 70 | | reduced | increased | hepato-splenomegaly |
| 11 | 67 | M | polycythaemia rubra vera | 10.9 | n.s. | 45 | ?0/320 | n.s. | reduced | yes |
| Mrs. S. P | 27 | F | menorrhagia thalassaemia trait | 5 6 | 59 | 77 | 70/480 13/86 µmol/l | reduced | normal | 2 cm |

n.s. = Not stated.
Mea results of 17 patients.
[illegible]

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| | 3 | M | pneumonia (premature baby) | 3.2 | 65 | 100 | n.s. | reduced | adequate | 2 cm |
| | 1 / 8 | F | fever cough (premature baby) | 3 8 | 61 | 197 | n.s. | reduced | normal | no |
| | 2 /12 | M | rhinorrhea (negro) | 4 0 | 54 | 50 | n.s. | not examined at presentation | | n.s. |
| | 1/1 | M | vomiting (premature baby) | 1 75 | 53 | 38 4 | n.s. | reduced | reduced | 2 cm |
| 1 | 4 | | hypersplenism[2] | 4 7 | 70 | 72 | 52/n.s. | n.s. | n.s. | yes |
| | | F | malabsorption | 5 6 | 71 | 145 | 46/n.s. | n.s. | n.s. | n.s. |
| | | F | malabsorption and chronic blood loss | 6.6 | 79 | 137 | 63/n.s. | n.s. | n.s. | n.s. |
| | | M | malabsorption | 6.5 | 77 | 121 | 36/n.s. | n.s. | n.s. | n.s. |
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| 10 | middle aged' | F | cirrhosis ascites 'hypersplenism | 7 1 | 98 | 70 | | reduced | increased | hepato-splenomegaly |
| 11 | 67 | M | polycythaemia rubra vera | 10 9 | n.s. | 45 | 20/320 | n.s. | reduced | yes |
| Mrs. S P | 27 | F | menorrhagia -thalassaemia trait | 5 6 | 59 | 77 | 70/480 13/86 µmol/l | reduced | normal | 2 cm |

n.s. = Not stated.

Mean results of 17 patients.

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# Fibrinogènes Québec I et Québec II: deux nouvelles familles de dysfibrinogénémie

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**Fibrinogen Quebec I and Quebec II: Two New Families of Dysfibrinogenemia**

*Key Words.* Clotting defect · Dysfibrinogenemia · Hemorrhagic diathesis

*Abstract.* Two new families of congenital dysfibrinogenemia originating from French Canada are reported. The dysfibrinogenemia in the first family is characterized by an abnormal aggregation of the fibrin monomers, the defect in the second family is due to a faulty release of fibrinopeptides during the proteolytic phase of the thrombin-fibrinogen reaction.

Nous rapportons nos observations sur deux familles canadiennes françaises atteintes de dysfibrinogénémie constitutionnelle.

## *Matériel et Méthodes*

Temps de saignement Ivy [3], rétraction du caillot [23], rétention plaquettaire [2], agrégation plaquettaire [1], consommation de la prothrombine [3]. Temps de céphaline activé: après avoir incubé pendant 10 min à 37 °C 0,2 ml de plasma citraté avec 0,2 ml de Platelin + activateur (Warner Lambert, N.J.), on recalcifie avec 0,2 ml de chlorure de calcium 0,025 M en Tris (Cl⁻) 0,05 M, pH 7,4. Temps de Quick [17] avec Simplastin (Warner-Lambert, N.J.). Dosage des facteurs II, V, VII, VIII, IX, X, XIII [3] (Warner Lambert, N.J.; Dade, Miami, Fla.). Temps de thrombine: au fibromètre on ajoute 0,1 ml de thrombine bovine (Parke-Davis, Détroit, Mich.), 5 U/ml de Tris salin, à 0,2 ml de plasma citraté. Temps de Reptilase: au fibromètre, on ajoute 0,1 ml de Reptilase (Sigma, St. Louis, Mo.), extrait de venin

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*Tableau I* Principaux tests de coagulation chez les 2 propositus

| | Valeurs normales | H G | L O |
|---|---|---|---|
| Temps de céphaline activé sec | 3 –45 | 36 | 47 |
| Temps de Quick, sec | 10,5 ± 1,0 | 10,5 | 14 |
| Temps de Quick d'un mélange à parties égales (normal + malade), sec | 10,5 | 10,5 | 11,5 |
| Facteurs II, V VII VIII IX, X, XIII | | normal | normal |
| Temps de thrombine, sec | 17 ± 2 | 25 | 24 |
| Temps de Reptilase, sec | 15 ± 1 | 21,5 | 23 |
| Fibrinogène | | | |
| Méthode colorimétrique, mg% | 200–400 | 265 | 280 |
| Titrage par la thrombine | 1/128 | 1/64 | 1/64 |
| Test FPT mg% | 175–375 | 174 | 130 |
| Méthode immunologique, mg % | 200–400 | 254 | 311 |
| Courbe de polymérisation de la fibrine | | anormale | anormale |
| Génération de thrombine | | normale | non fait |
| Antithrombine III | | | |
| Activité biologique | | normale | normale |
| Méthode immunologique, mg% | 25–35 | 29 | 27 |
| Antigène FR dans le sérum | | | |
| Méthode au latex, µg/ml | 8 | 2 | 220 |
| Agglutination des staphylocoques | 1/1 | 1/1 | 1/18 |

dominant. Dans la famille G 7 des 9 membres étudiés sont porteurs de la tare, tandis que dans la famille O 6 des 9 sujets étudiés sont atteints.

L analyse de l'allongement des temps de thrombine et de Reptilase des 2 propositus permet d'observer des corrections partielles par le chlorure de calcium, par le sulfate de protamine par l'abaissement de la force ionique et par une concentration plus grande de thrombine ou de Reptilase Le plasma défibriné et le sérum n allongent pas les temps de thrombine et de Reptilase d'un plasma normal.

L étude spectrophotométrique de la polymérisation de la fibrine provoquée en plasma citraté par la thrombine ou la Reptilase (fig. 1a,b) montre chez H G une absence de variation de la densité optique malgré l'obtention d'un caillot et, chez L O un retard et une lenteur anormale de la polymérisation avec cependant une densité optique finale en bon accord avec le taux de fibrinogène. Le résultat observé pour H. G corres-

de *Botrops athrox* à 0,2 ml de plasma citraté [21]. Courbe de polymérisation de la fibrine [21] Dosage du fibrinogène· méthode colorimétrique [25] titrage par la thrombine [20] test FPT (fibrin polymerization time) [24] méthode immunologique [14]. Immuno-électrophorèse du fibrinogène [9] (Behringwerke, Marburg/Lahn, RFA) Test de génération de thrombine [4]. Antithrombine III mesure de l'activité biologique [8] dosage immunologique [14]. Détermination du fibrinogène (antigène FR) dans le sérum méthode au latex [3] avec le Thrombo-Wellcotest (Warner Lambert, NJ) agglutination des staphylocoques [3] (Behringwerke, Marburg/Lahn, RFA) Temps de lyse des euglobulines plasmatiques [16] Dosage du plasminogène et de la plasmine active par la méthode des plaques de fibrine (Hyland, Calif.).

Le fibrinogène humain est purifié selon REGOECZI [18]. Etude de la libération des fibrinopeptides par la thrombine d'après la technique originale de LORAND [11] modifiée [7] les fibrinopeptides sont libérés par l action de la thrombine sur une solution de fibrinogène purifié et sont dosés par la méthode de LOWRY *et al.* [12] avec la modification suivante [7] pour neutraliser l excès d'acidité des échantillons, la concentration du NaOH dans le réactif A est doublée

Agrégation des monomères de fibrine selon la méthode de FERRY et MORRISON [6] modifiée [5] une solution de monomères de fibrine est préparée avec de la thrombine bovine et de la Reptilase à partir de sang prélevé sur EDTA disodique

## *Résultats*

Famille G la découverte de l anomalie y fut fortuite le propositus H. G., servit de «témoin normal» pour des épreuves de coagulation! Il n existe pas de diathèse hémorragique dans cette famille

Famille O l anomalie fut découverte à l occasion d une investigation du propositus, L. O qui avait présenté une insuffisance respiratoire prolongée après anesthésie générale. On découvrit un déficit modéré de la pseudo-cholinestérase, qu on considéra responsable de cette hypoventilation anormale La diathèse hémorragique des membres de la fratrie immédiate du propositus est très légère toutefois, ils n ont pratiquement pas subi d'interventions chirurgicales Par ailleurs, 2 cousines du côté paternel et leur père sont décédés d'hémorragie dans les suites d'une amygdalectomie A l époque, la tare familiale n était pas connue faute de survivants de cette lignée de la famille, l anomalie n a pu y être recherchée.

Les principaux résultats des tests de coagulation des deux propositus (tabl I) montrent qu il existe une anomalie à l étape de la fibrinoformation. Les fonctions plaquettaires et la fibrinolyse sont normales. Chez L. O., on remarquera le test FPT anormal, la présence d antigène FR dans le sérum et l allongement du temps de Quick

L étude des deux familles a montré un mode de transmission autosome

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## *Résultats*

Famille G la découverte de l anomalie y fut fortuite le propositus H G servit de «témoin normal» pour des épreuves de coagulation! Il n existe pas de diathèse hémorragique dans cette famille

Famille O l anomalie fut découverte à l occasion d une investigation du propositus, L. O qui avait présenté une insuffisance respiratoire prolongée après anesthésie générale. On découvrit un déficit modéré de la pseudo-cholinestérase qu on considéra responsable de cette hypoventilation anormale. La diathèse hémorragique des membres de la fratrie immédiate du propositus est très légère toutefois, ils n ont pratiquement pas subi d interventions chirurgicales. Par ailleurs, 2 cousines du côté paternel et leur père sont décédés d hémorragie dans les suites d une amygdalectomie. A l époque la tare familiale n était pas connue faute de survivants de cette lignée de la famille, l anomalie n a pu y être recherchée.

Les principaux résultats des tests de coagulation des deux propositus (tabl I) montrent qu il existe une anomalie à l étape de la fibrinoformation. Les fonctions plaquettaires et la fibrinolyse sont normales. Chez L. O., on remarquera le test FPT anormal la présence d antigène FR dans le sérum et l allongement du temps de Quick

L étude des deux familles a montré un mode de transmission autosome

*Tableau I* Principaux tests de coagulation chez les 2 propositus

| | Valeurs normales | H. G | L. O |
|---|---|---|---|
| Temps de céphaline activé, sec | 37–45 | 36 | 47 |
| Temps de Quick, sec | 10,5 ± 1,0 | 10,5 | 14 |
| Temps de Quick d'un mélange à parties égales (normal + malade), sec | 10,5 | 10,5 | 11,5 |
| Facteurs II, V VII VIII, IX, X, XIII | | normal | normal |
| Temps de thrombine, sec | 17 ± 2 | 25 | 4 |
| Temps de Reptilase, sec | 15 ± 1 | 21,5 | 23 |
| Fibrinogène | | | |
| Méthode colorimétrique, mg% | 200–400 | 265 | 280 |
| Titrage par la thrombine | 1/128 | 1/64 | 1/64 |
| Test FPT mg% | 175–375 | 174 | 130 |
| Méthode immunologique mg% | 200–400 | 254 | 311 |
| Courbe de polymérisation de la fibrine | | anormale | anormale |
| Génération de thrombine | | normale | non fait |
| Antithrombine III | | | |
| Activité biologique | | normale | normale |
| Methode immunologique, mg% | 25–35 | 29 | 27 |
| Antigène FR dans le sérum | | | |
| Methode au latex, µg/ml | 8 | 2 | <20 |
| Agglutination des staphylocoques | 1/1 | 1/1 | 1/18 |

dominant. Dans la famille G  7 des 9 membres étudiés sont porteurs de la tare, tandis que dans la famille O 6 des 9 sujets étudiés sont atteints.

L analyse de l'allongement des temps de thrombine et de Reptilase des 2 propositus permet d observer des corrections partielles par le chlorure de calcium, par le sulfate de protamine, par l'abaissement de la force ionique et par une concentration plus grande de thrombine ou de Reptilase. Le plasma défibriné et le sérum n allongent pas les temps de thrombine et de Reptilase d'un plasma normal

L étude spectrophotométrique de la polymérisation de la fibrine provoquée en plasma citraté par la thrombine ou la Reptilase (fig. 1a,b) montre chez H. G  une absence de variation de la densité optique malgré l'obtention d'un caillot et, chez L. O un retard et une lenteur anormale de la polymérisation avec cependant une densité optique finale en bon accord avec le taux de fibrinogène La résultat observé pour H. G corres-

de *Botrops athrox* à 0,2 ml de plasma citraté [21]. Courbe de polymérisation de la fibrine [21]. Dosage du fibrinogène: méthode colorimétrique [25]; titrage par la thrombine [20]; test FPT (fibrin polymerization time) [24]; méthode immunologique [14]. Immuno-électrophorèse du fibrinogène [9] (Behringwerke, Marburg/Lahn, RFA). Test de génération de thrombine [4]. Antithrombine III: mesure de l'activité biologique [8]; dosage immunologique [14]. Détermination du fibrinogène (antigène FR) dans le sérum: méthode au latex [3] avec le Thrombo-Wellcotest (Warner-Lambert, N.J.); agglutination des staphylocoques [3] (Behringwerke, Marburg/Lahn, RFA). Temps de lyse des euglobulines plasmatiques [16]. Dosage du plasminogène et de la plasmine active par la méthode des plaques de fibrine (Hyland, Calif.).

Le fibrinogène humain est purifié selon REGOECZI [18]. Etude de la libération des fibrinopeptides par la thrombine d'après la technique originale de LORAND [11] modifiée [7]: les fibrinopeptides sont libérés par l'action de la thrombine sur une solution de fibrinogène purifié et sont dosés par la méthode de LOWRY *et al.* [12] avec la modification suivante [7]: pour neutraliser l'excès d'acidité des échantillons, la concentration du NaOH dans le réactif A est doublée.

Agrégation des monomères de fibrine selon la méthode de FERRY et MORRISON [6] modifiée [5]: une solution de monomères de fibrine est préparée avec de la thrombine bovine et de la Reptilase à partir de sang prélevé sur EDTA disodique 2%.

## *Résultats*

Famille G.: la découverte de l'anomalie y fut fortuite: le propositus H.G. servit de «témoin normal» pour des épreuves de coagulation! Il n'existe pas de diathèse hémorragique dans cette famille.

Famille O.: l'anomalie fut découverte à l'occasion d'une investigation du propositus L.O. qui avait présenté une insuffisance respiratoire prolongée après anesthésie générale. On découvrit un déficit modéré de la pseudo-cholinestérase qu'on considéra responsable de cette hypoventilation anormale. La diathèse hémorragique des membres de la fratrie immédiate du propositus est très légère; toutefois, ils n'ont pratiquement pas subi d'interventions chirurgicales. Par ailleurs, 2 cousines du côté paternel et leur père sont décédés d'hémorragie dans les suites d'une amygdalectomie. A l'époque, la tare familiale n'était pas connue faute de survivants de cette lignée de la famille: l'anomalie n'a pu y être recherchée.

Les principaux résultats des tests de coagulation des deux propositus (tabl. I) montrent qu'il existe une anomalie à l'étape de la fibrinoformation. Les fonctions plaquettaires et la fibrinolyse sont normales. Chez L.O. on remarquera le test FPT anormal, la présence d'antigène FR dans le sérum et l'allongement du temps de Quick.

L'étude des deux familles a montré un mode de transmission autosome

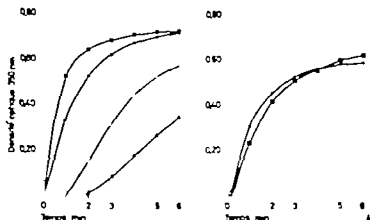



*Fig* 2. Agrégation des monomères de fibrine purifiés de L.O. préparés avec la thrombine bovine. Concentration finale de la solution de monomères 40 mg%, pH final 6,4 forces ioniques finales 0,05 et 0,15 température 25 C.
■ = Sujet normal, force ionique 0,05 ● = L.O force ionique 0,05 O = sujet normal, force ionique 0,15 ▲ = L.O force ionique 0,15. *b* Agrégation des monomères de fibrine purifiés de L.O préparés avec la Reptilase. Concentration finale de la solution de monomères. 40 mg%, pH final 6,4 force ionique finale. 0,05 température 25 °C. ■ = Sujet normal ● = L.O

que 0 15 et diminuée pour H. G (fig. 3a, b). L étude cinétique de la libération des fibrinopeptides par l'action de la thrombine sur le fibrinogène purifié est résumée dans le tableau II La vitesse et le taux de libération des fibrinopeptides du fibrinogène H. G sont normaux, tandis qu'ils sont diminués chez le propositus L. O et sa sœur G O

L'immuno-électrophorèse du fibrinogène dans le plasma ne montre aucune anomalie chez les 2 propositus. Chez L. O on observe la présence de fibrinogène dans le sérum. L étude du caillot de H G. en microscopie à balayage a montré que les filaments de fibrine formés par la thrombine calcique en plasma citraté, à pH 7 4 et force ionique 0 15 sont plus fins et plus fragmentés que ceux d'un plasma normal (fig. 4a, b).

## *Discussion*

Notre investigation a révélé une dysfonction de la fibrinoformation chez 13 des 18 sujets étudiés dans ces deux familles qui est due à une

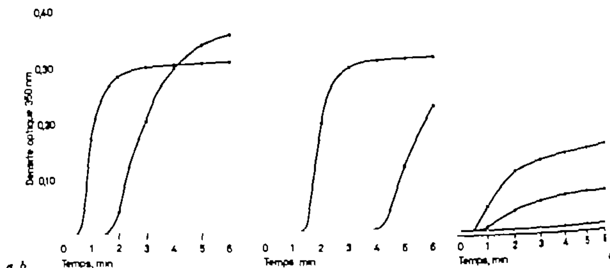



*Fig 1* *b* Courbes de polymérisation de la fibrine induite par la thrombine bovine (0,5 U/ml Tris salin, concentration finale a) et la Reptilase, (0,06 unités Klobusitzky/ml Tris salin, concentration finale b). Plasma dilué au dixième en NaCl 0,15 M. Température 25 C. ■ = Plasma normal ● = plasma L. O O = plasma H G *c* Effet du plasma H G sur la polymérisation de la fibrine d'un plasma normal par la Reptilase. ■ = Plasma normal ● = plasma normal + tampon Tris salin à parties égales O = plasma normal + plasma H G à parties égales.

*Tableau II* Etude cinétique de la libération des fibrinopeptides pour 3 fibrinogènes normaux et 3 fibrinogènes anormaux

| | Fibrinopeptides libérés par milligramme de fibrinogène, µg | | | | | |
|---|---|---|---|---|---|---|
| | 1 min | min | 5 min | 10 min | 20 min | 30 min |
| Fibrinogène normal | | | | | | |
| 1 | 8 8 | 13,4 | 15,9 | 17 8 | 19,[illegible] | 20,1 |
| | 10,0 | 15,2 | 17,[illegible] | 19,2 | 20 6 | 19,5 |
| 3 | 8,5 | 13 4 | 16,2 | 18 7 | 20,9 | 20,3 |
| Fibrinogène H G | 12,6 | 13 7 | 16 2 | 18,9 | 19 5 | 18,9 |
| Fibrinogène L. O | 3,5 | 6,6 | 11 2 | 1[illegible],3 | 13 2 | 13, |
| Fibrinogène G O | 4 6 | 7 1 | 8,8 | 11,2 | 12,6 | 13 7 |

pond très bien au fait que les caillots formés en plasma H G produisent un gel remarquablement translucide Le plasma H G perturbe de façon importante la courbe de polymérisation d un plasma normal (fig 1c)

L agrégation des monomères de fibrine préparés avec la thrombine ou la Reptilase est légèrement anormale pour L. O (fig 2a, b) à force ioni-

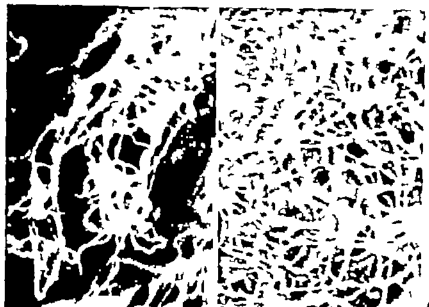



*Fig. 4* Structure du caillot en microscope à balayage de champs. Plasma citraté; thrombine bovine 5 U/ml de $CaCl_2$ 0,025 M (concentration finale). Caillots fixés dans la solution de Rdeu ?u *et al* [19] et séchés par le point critique × 10,000 Plasma normal. *b* Plasma H O.

L. O n ayant pas de diathèse hémorragique familiale peut conduire à envisager la possibilité d'une coagulation intravasculaire disséminée ou d'une affection hépatique sévère. Toutefois, il s agissait pour L. O d'une fausse hypofibrinogénémie qui n était retrouvée qu avec les méthodes de dosage basées sur la vitesse de coagulation du plasma (test FPT). Cette discordance des taux selon les méthodes a déjà été observée [13 15]. Le caillot plasmatique obtenu chez H G ne causait pratiquement pas d'augmentation de la densité optique du plasma, comme dans le cas des fibrinogènes Montréal [10] et Nancy [22]

Les études en vue de préciser l anomalie fonctionnelle de la molécule de fibrinogène ont identifié jusqu à maintenant trois grands types d'anomalie [13] a) une anomalie de la libération des fibrinopeptides (phase protéolytique) b) une anomalie de l agrégation des monomères de fibrine (phase de polymérisation) et c) une anomalie de la stabilisation des polymères. Nos études ont montré que dans la famille O il s agissait d'une

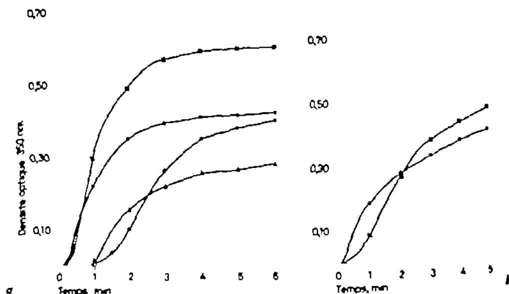



*Fig 3 a* Agrégation des monomères de fibrine purifiés de H.G préparés avec la thrombine bovine. Concentration finale de la solution de monomères: 40 mg%, pH final 6,4 forces ioniques finales 0,07 et 0 11 température 25 C.

■ = Sujet normal, force ionique 0,07 ● = H G force ionique: 0,07 O = sujet normal force ionique 0,11 ▲ = H G force ionique 0,11 *b* Agrégation des monomères de fibrine purifiés de H G préparés avec la Reptilase. Concentration finale de la solution de monomères: 40 mg%, pH final 6,4 force ionique finale 0,025 température 25 °C, ■ = Sujet normal ● = H G

anomalie qualitative de la molécule de fibrinogène. Une revue des familles rapportées [13 15] montre que les temps de Quick et de céphaline activé sont soit normaux soit allongés ils sont normaux dans la famille G et allongés dans la famille O

La diathèse hémorragique est nulle dans la famille G et légère chez les membres vivants de la famille O Mais 3 autres membres de la famille O qui n ont pu être étudiés, sont décédés d'hémorragie peu après une amygdalectomie La prudence doit donc nous faire craindre des hémorragies graves au moment d actes chirurgicaux dans de tels cas. Parmi les 26 familles publiées en 1974 [13 15] 13 n avaient pas de diathèse hémorragique et les 13 autres avaient une diathèse hémorragique légère.

L immuno-électrophorèse du sérum de L. O a révélé une molécule semblable au fibrinogène qui se traduisait par un taux élevé d antigène FR dans le sérum de cette patiente. L association d un taux élevé d antigène FR sérique à une hypofibrinogénémie chez une adulte, comme

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libération retardée des fibrinopeptides. Par contre, dans la famille G nous avons observé une vitesse normale de libération des fibrinopeptides mais une agrégation des monomères nettement réduite en cela également, ce fibrinogène ressemble au fibrinogène Montréal [10] Nous proposons de désigner ces fibrinogènes anormaux du nom de fibrinogène Québec I pour la famille G et de fibrinogène Québec II pour la famille O

## *Résumé*

Deux familles canadiennes françaises atteintes de dysfibrinogénémie congénitale sont rapportées. La première dysfibrinogénémie est caractérisée par un déficit de l agrégation des monomères de fibrine la seconde est due à une anomalie de la libération des fibrinopeptides.

## *Remerciements*

Les auteurs remercient le docteur ROBERT GUIDOIN qui a effectué les études des caillots en microscopie électronique à balayage.

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Acta haemat. 59: 129–143 (1978)

# Familiärer Myeloperoxidasedefekt und akute myeloische Leukämie

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## Familial Peroxidase-Deficiency and Acute Myeloic Leukemia

*Key Words.* Myeloperoxidase deficiency · Acute myeloic leukemia · Primary neutrophil granules

*Abstract.* A complete lack of myeloperoxidase (MPO) was demonstrated in a boy suffering from acute myeloic leukemia during the acute phase of the disease and after a remission was achieved. A partial defect of MPO was demonstrated in the patient's father; no further abnormalities were seen in other members of the family. The fine structure of the patient's neutrophils and monocytes appeared normal, no activity of MPO was demonstrated on the fine structural level. In the father's neutrophils transitional forms between cells exhibiting a normal MPO activity and those without activity were demonstrated. The neutrophil bactericidal activity was strongly inhibited in the patient and decreased in his father. Normal values were found for NBT test, chemotaxis, serum-dependent phagocytosis, number of B and T lymphocytes, serum immunoglobulins, and complement. A possible connection between MPO deficiency and leukemia is discussed.

Myeloperoxidase ist ein lysosomales Enzym der neutrophilen Granulozyten und der Monozyten, dem Bedeutung bei der Abtötung phagozytierter Mikroorganismen zukommt [15]. Ein völliger Mangel an MPO wurde als autosomal-rezessiv erblicher Enzymdefekt in bisher 7 Fällen

Myeloperoxidase = MPO; Naphthol-AS-Azetat-Esterase = NAS; Naphthol-ASD-Chlorazetat-Esterase = ASDCl; Nitroblautetrazolium = NBT; Perjodsäure-Schiffs-Reagenz = PAS

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H. Kief (ed.) **Iron Metabolism and its Disorders.** Workshop Conferences Hoechst, vol. 33. Excerpta Medica, Amsterdam 1975 X + 346 pp. Dfl. 80.– / US $ 31.95. ISBN 90-219-0300-8.

This volume summarises presentations and discussions of an international meeting held in April 1975 in Germany. The book is divided into four sections concerning iron balance, iron deficiency, parenchymal iron overload (including sideroblastic anemias) and principles of therapy (iron substitution or treatment of iron overload). The book contains up-to-date summaries by leading experts. New information could be detected in the original publications, but most chapters serve their purpose of selective reviewing well. Personally I am going to use quite a few pictures for teaching purposes (all reproductions are carefully presented). More recent studies which have not yet been covered in similar reviews concern the structure and functions of ferritin, red cell metabolism of pyridoxine in various types of anemia and detailed morphologic studies of abnormal sideroblasts. Citation of key references has been handled unevenly and quotations are without titles (a notoriously poor habit in reviews). The discussion has not been reedited. All in all I find this book useful.

E. A. Beck, *Bern*

T. H. Spaet (ed.) **Progress in Hemostasis and Thrombosis**, vol. 3. Grune & Stratton, New York 1976. XVI + 342 pp. US $ 31.50. ISBN 0-8089-0939-8.

The 3rd volume of *Progress in Hemostasis and Thrombosis* edited by T. H. Spaet in 1976, brings a new series of reviews. The chosen subjects are of variable value. One outstanding contribution has been written by Mosesson and Finlayson on the search for the structure of fibrinogen. This contribution delineates the state of our knowledge on fibrinogen, the hypotheses, the questionable data and the direction of research. Other chapters constitute purely a collection of informations from the literature, for example, the chapter of Bellamarich on Hemostasis in Animals other than Mammals. The Role of Cells. This review gives a summary on the available informations, but not more. The more practical part of this volume 3 is reserved to the 'Control of Heparin Therapy' due to Wessler and Gitel. However the title is misleading since practically all aspects of heparin therapy are considered except the control. Some statements are manifestly too optimistic. The demonstration that the low dose heparin therapy is free of bleeding has not really been done, in each series there is a certain number of bleeding complications. One misses in this review a clear indication of the very frequently important prolongation of the thrombin time by low doses of heparin. This transitory effect can reach thrombin time values comparable to those of full heparinization.

Despite some imperfections, this book can be recommended to people working or interested in coagulation, platelets and heparin treatment who will find here current information.

F. Duckert, *Basel*

*Elektronenmikroskopische Zytochemie* Peroxidaseaktivität wurde in für die elektronenmikroskopische Untersuchung präparierten Zellsuspensionen mit Diaminobenzidin und $H_2O_2$ nachgewiesen [13].

*Untersuchungen zur Granulozytenfunktion In-vitro*-Phagozytose und intrazelluläre Abtötung eines pathogenen *Staphylokokkus-aureus*-Stammes (502 A) wurden nach der Methode von QUIE *et al* [22] untersucht. Der *VBT Test* wurde nach der Beschreibung von PARK *et al* [20] sowie in der Modifikation nach PARTSCH und HITZIG [21] durchgeführt. Die *chemotaktische* Antwort der Granulozyten auf Casein als chemotaktischer Substanz wurde in modifizierten Boyden-Kammern nach der methodischen Beschreibung von WILKINSON [33] untersucht.

Die *quantitative Bestimmung der B- und T Lymphozyten* erfolgte durch den Nachweis von Oberflächenrezeptoren für Komplement und Immunglobuline bzw für unvorbehandelte Schaferythrozyten [14]. Kommerziell verfügbare Immundiffusionsplatten (Partigen Behringwerke, Marburg) dienten zur *quantitativen Bestimmung der Immunglobuline* und der *Komplementkomponenten C3 C4 und C5*

## Befunde

### Lichtmikroskopie und Zytochemie

*Patient B H* Das Knochenmark ist dicht infiltriert durch wenig differenzierte *Blasten*. Die Kerne dieser Zellen zeigen ein lockeres Chromatin, 1–2 Nukleolen, sie sind z.T etwas eingebuchtet oder angedeutet gelappt das mittelblaue Zytoplasma enthält meistens keine selten einige azurophile Granula. Aktivität von Peroxidase oder Sudanschwarz B-anfärbbares Material sind in den Blasten nicht festzustellen. Hingegen zeigen einige der wenig differenzierten atypischen Zellen eine schwache, selten eine ausgeprägtere Aktivität von ASDCl oder (NaF-hemmbarer) NAS und damit eine beginnende Differenzierung zu Promyelozyten bzw zu Promonozyten. Die *neutrophilen Granulozyten* enthalten im leukämischen Stadium Doehle Körper häufig besteht eine Pelgersche Kernanomalie. Bei späteren Untersuchungen, als eine weitgehende hämatologische Teilremission besteht, sind die Granulozyten in ihrer Morphologie unauffällig. Bei mehreren Kontrollen in verschiedenen Phasen der Erkrankung sind Neutrophile und Monozyten vollständig Peroxidase- und Sudanschwarz B-negativ Die übrigen Enzyme sind in normalen Konzentrationen nachzuweisen. *Eosinophile* sind morphologisch und zytochemisch normal (Tab. I).

*Verwandte des Patienten W H* Bei allen Verwandten sind die Blutzellen zahlenmässig und morphologisch unauffällig. Allerdings besitzen beim Vater nur weniger als die Hälfte der neutrophilen Granulozyten eine normale Konzentration von Peroxidase und von Sudanschwarz B die restlichen Neutrophilen sowie die grosse Mehrzahl der Monozyten sind Peroxi-

beobachtet [16 23 32] Häufiger lässt sich ein partieller Peroxidasemangel bei Patienten mit unreifzelligen myeloischen Leukämien feststellen [4 9 11]

Derartige Defektzustände bieten Gelegenheit die physiologische Bedeutung des MPO-Systems zu untersuchen sie erlauben Rückschlüsse auf die normale Entwicklung myeloischer Zellen und ihrer Granula sie sind ein Beispiel für die gestörte Zelldifferenzierung im Rahmen einer Leukämie

Wir beobachteten das vollständige Fehlen der MPO in den Neutrophilen und Monozyten eines 15jährigen Jungen mit unreifzelliger myeloischer Leukämie beim Vater des Patienten wurde ein partieller Defekt dieses Enzyms nachgewiesen Klinische Befunde sowie morphologische und funktionelle Untersuchungen die an den Leukocyten des Patienten und seines Vaters durchgeführt wurden sollen mitgeteilt werden

## Kasuistik

*Patient* W H geb Nov 1961 Als Kleinkind mehrmals eitrige Otitis media und Angina tonsill Tonsillektomie. Als Schulkind rezidivierende Sinubronchitiden. Im März 1976 Haematome Blässe, eitrige Rhinitis. Stationäre Aufnahme in der Kinderklinik d. Univ München und Diagnose einer unreifzelligen myeloischen Leukämie. Befunde bei der Aufnahme. Milz, Leber und Lymphknoten palpatorisch kaum vergrössert. Hb 5 8 g%, Retikulozyten 3‰ Thrombozyten 7000/mm³ Leukozyten 38000/mm³ davon etwa 50% wenig differenzierte Blasten. Im Sternalmark dichte Infiltration mit polymorphen Blasten, die nur z.T eine beginnende Differenzierung in die granulozytäre oder monozytäre Reihe zeigten. Im weiteren Verlauf traten komplizierend Bronchopneumonien und ein ausgedehnter Soorbefall der Mundschleimhäute auf Unter zytostatischer Behandlung mit 6-Mercaptopurin Vincristin und Adriblastin, in Verbindung mit Glukokortikoiden, konnte schliesslich eine weit gehende hämatologische Teilremission erreicht werden.

*Familienangehörige* Beide Eltern, d e Grossmutter mütterl cherseits, zwei Stiefschwestern des Vaters und die einzige Schwester des Patienten wurden untersucht Alle Personen waren klinisch und hämatologisch unauff llig lediglich beim *Vater* wurde ein *partieller* Myeloperoxidasedefekt nachgewiesen.

## Methodik

*Zytochemi* Lichtmikroskopischer Nachwel von Peroxidase [26] Sudanschwarz B [30] saurer Phosphatase [6] N phthol AS-Azetat E terase und *Hemmung durch* NaF [18, 28] Naphthol AS D-Chlorazetat Esterase [19] PAS [7].




*Abb. 1.* Neutrophiler Granulozyt des Patienten W. H. Nachweis endogener Peroxidase: Granula, welche Peroxidase enthalten, müssten besonders dunkel dargestellt sein. Der Schnitt wurde aber mit Bleihydroxid-Uranylazetat kontrastiert, so dass alle Strukturen, auch die Peroxidase-negativen Granula, deutlich dargestellt sind. Bei sonst normaler Feinstruktur enthält die Zelle grosse, homogene Zytoplasmaeinschlüsse (×) sowie Vakuolen (Pfeile). In beiden Fällen dürfte es sich um Zytosomen, bzw. Vakuolen handeln, die teils phagozytiertes Material enthalten (Phagolysosomen), teils autolytisch bedingt sind (Autophagolysosomen).

dase und Sudanschwarz B-negativ. Die Eosinophilen des Vaters sowie alle Blutzellen der übrigen Verwandten sind zytochemisch unauffällig (Tab. 1).

*Elektronenmikroskopie*

Die Leukozyten des Patienten W. H. und seines Vaters zeigen eine normale *Feinstruktur*. Die Granula der Granulozyten und Monozyten ent

*Tabelle 1* Zytochemische Befunde von Blutausstrichen des Patienten und seiner Familienangehörigen vom April bis November 1976

| | W H<br>Leukämie in Teilremission<br>(7 Kontrollen April bis Nov 1976) | | | J H<br>Vater des Patienten<br>(2 Kontrollen Mai Juli 1976) | | | 5 weitere Verwandte<br>des Patienten<br>(2 Kontrollen Mai Juli 1976) | | |
|---|---|---|---|---|---|---|---|---|---|
| | neutr Gran. | Mono. | Eos. | neutr Gran. | Mono | Eos. | neutr Gran. | Mono. | Eos. |
| Peroxidase | 100% – | 100% – | 100% ++ | 41% +/++<br>38% (+)<br>21% – | 21% (+)<br>79% – | 100% ++ | 98–100% ++ | 50–75% (+) | 100% ++ |
| Sudan B | – | – | | –/+ | –/(+) | | | | |
| ASD Cl | +/++ | –/(+) | – | +/++ | –/(+) | – | +/++ | –/(+) | – |
| NAS | (+) | ++ | (+) | (+) | ++ | (+) | (+) | ++ | (+) |
| a. Phosph. | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| PAS | + | + | + | + | + | + | + | + | + |




*Abb 1* Neutrophiler Granulozyt des Patienten W H Nachweis endogener Peroxidase: Granula, welche Peroxidase enthalten, mussten besonders dunkel dargestellt sein. Der Schnitt wurde aber mit Bleihydroxid-Uranylazetat kontrastiert, so dass alle Strukturen, auch die Peroxidase-negativen Granula, deutlich dargestellt sind. Bei sonst normaler Feinstruktur enthält die Zell grosse, homogene Zytoplasmaeinschlüsse (X) sowie V kuolen (Pfeile) I beiden Fallen durfte es sich um Zytosomen, bzw Vakuolen handeln, die teils phagozytiertes Material enthalten (Phagolysosomen), teils autolytisch bedingt sind (Autophagolysosomen)

dase und Sudanschwarz B-negativ Die Eosinophilen des Vaters sowie alle Blutzellen der übrigen Verwandten sind zytochemisch unauffällig (Tab I)

*Elektronenmikroskopie*

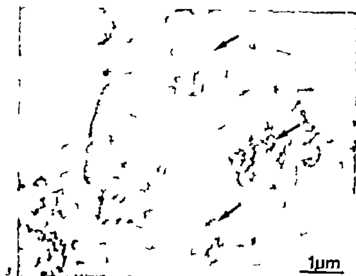
Die Leukozyten des Patienten W H. und seines Vaters zeigen eine normale *Feinstruktur* Die Granula der Granulozyten und Monozyten ent




*Abb* Monozyt des Patienten W H Präparation und Schnittkontrastierung wie Abbildung 1 Auch dieser Monozyt enthält grosse Zytoplasmaeinschlüsse und Vakuolen (X).

sprechen in Grösse und Form denen gesunder Personen Die Neutrophilen (Abb 1) und die Monozyten (Abb 2) von W H enthalten oft Einschlüsse phagozytierten Materials und grosse Vakuolen

*Endogene Peroxidase* Aktivität endogener Peroxidase fehlt auch im elektronenmikroskopischen Präparat in den Neutrophilen (Abb 3) und Monozyten von W H während sie in den Eosinophilen in normaler Stärke vorhanden ist (Abb 4) Beim Vater des Patienten finden sich nebeneinander Neutrophile mit fehlender mit schwach ausgeprägter und mit normaler Peroxidaseaktivität. Bei Neutrophilen mit schwach ausgeprägter Aktivität sind erstens weniger Granula in einer Zelle angefärbt, zweitens ist die Anfärbung des einzelnen positiven Granulums schwächer (Abb 5) Einige der Monozyten des Vaters von W H enthalten kleine Peroxidase positive Granula, andere sind negativ



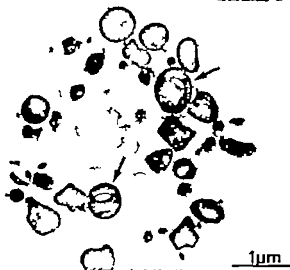




*Abb 3* Neutrophiler Granulozyt des Patienten W H Keine Schnittkontrastierung Peroxidaseaktivität ist nicht nachzuweisen Vakuolen wie in Abbildung 1

*Abb 4* Eosinophiler Granulozyt des Patienten W H Keine Schnittkontrastierung. Normale Peroxidaseaktivität der spezifischen Granula, die z.T an den kristalloiden Einschlüssen zu erkennen sind (Pfeile).




*Abb* Monozyt des Patienten W H Präparation und Schnittkontrastierung wie Abbildung 1 Auch dieser Monozyt enthält grosse Zytoplasmaeinschlüsse und Vakuolen (X).

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*Abb. 6* Repräsentatives Testergebnis der granulozytären Bakterienabtötung. Zur Verwendung kamen 25% autologes Serum und *S. aureus* (A 502). Der Zusatz gepoolter Normalseren brachte keine Änderung des Kurvenverlaufs.

abhängige *Phagozytose* des pathogenen *S. aureus* Stammes (A 502) war beim Patienten, seinem Vater und einer gesunden Kontrollperson nicht gestört. Dies konnte durch Kontrolluntersuchungen bestätigt werden.

Eindeutig pathologische Ergebnisse zeigten die folgenden *Granulozyten-Funktionsuntersuchungen*. Die intrazelluläre *Abtötung* der aufgenommenen Bakterien (Abb. 6) war bei dem Patienten defekt. Nach 60 und 120 min zeigte sich keine signifikante Abnahme der zum Zeitpunkt 0 eingesäten Bakterienzahl. Auch durch Serumzugaben gesunder Kontrollpersonen konnte dieser Bakterien-Killing-Defekt nicht ausgeglichen werden. Die aus dem peripheren Blut des Vaters isolierten Granulozyten waren bei insgesamt vier Testansätzen zu verschiedenen Zeitpunkten nicht in der Lage, die Abtötungsleistung einer gesunden Kontrollperson zu erbringen (Abb. 6 – es muss auf die logarithmische Darstellung geachtet werden – die bakterizide Leistung der Granulozyten des Vaters erreicht nicht 50% der Normalkontrolle). Damit ist ein Defekt der granulozytären Bakterienabtötung nachgewiesen, der mit dem zytochemischen MPO-Mangel zu vereinbaren ist. Alle anderen Familienmitglieder (Patientenmutter




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*Granulozytenfunktion*

Für alle Familienmitglieder (Patient Eltern Schwester) fielen der *NBT Test* wie auch der *stimulierte NBT Test* wiederholt normal aus. Ein Defekt in der *chemotaktischen Leistung der Granulozyten* des Patienten und der direkten Familienmitglieder war nicht nachweisbar Die serum-




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erkrankten Sohn fehlte MPO vollkommen. Ein vollständiger MPO-Verlust wurde jedoch als erworbener Defekt im Rahmen einer Leukämie niemals beobachtet. Es können daher die beiden folgenden Möglichkeiten in Betracht gezogen werden. 1. Bei unserem Leukämiepatienten bestand ein konstitutioneller und partieller MPO-Defekt, zu dem eine Leukämie-bedingte Störung hinzukam, so dass ein vollständiges Fehlen des Enzyms resultierte. 2. Bei unserem Patienten bestand von vornherein ein konstitutioneller und vollständiger MPO-Defekt. In beiden Fällen kann diskutiert werden, ob der Enzymdefekt zum Auftreten einer Leukämie prädisponierte. Zusätzlich bleibt die Frage offen, ob unter den zahlreichen Fällen von erworbenem Peroxidasedefekt bei unreifzelligen myeloischen Leukämien nicht auch Patienten enthalten sind, bei denen vor dem Ausbruch der Leukämie bereits ein konstitutioneller, unerkannter MPO-Defekt bestand.

*Klinisch* erscheinen Patienten mit vollständigem MPO-Defekt unauffällig [32] oder zeigen eine *erhöhte Infektanfälligkeit*, z.B. gegenüber *Candida albicans* [16]. Bei unserem Leukämiepatienten ist anamnestisch eine vermehrte Infektanfälligkeit zu vermuten, die Anamnese des Vaters ist in dieser Hinsicht unergiebig.

*In vitro* lässt sich bei vollständigem Peroxidasedefekt eine Störung der *Abtötung* von durch Neutrophile *phagozytierten Bakterien (Staphylococcus aureus, Serratia marcescens)* und von Candida nachweisen [16], die aber nicht das Ausmass wie bei der chronischen septischen Granulomatose erreicht. Bei den von uns untersuchten Fällen entsprach das Ausmass des MPO-Defektes der *in vitro* nachgewiesenen Störung der Abtötung phagozytierter Bakterien. Die Abtötung phagozytierter Mikroorganismen wird bei MPO-Defekt jedoch nur verlangsamt, nicht aufgehoben. Das MPO-$H_2O_2$-System scheint demnach nicht die alleinige und unersetzliche Voraussetzung für eine ausreichende Bakterienabtötung zu sein [15]. Durch immunologische Methoden (Geldiffusion, Präzipitation, Immunofluoreszenz) unter Verwendung von Anti-Peroxidase-Antikörpern wurde beim konstitutionellen MPO-Defekt das Fehlen des Enzymmoleküls nachgewiesen [24]. Es handelt sich somit bei dieser Störung nicht um die Synthese eines lediglich funktionell defekten Enzyms.

Die *primären Granula* der Neutrophilenreihe sind bei konstitutionellem *MPO-Defekt normal angelegt. Sie unterscheiden sich in ihrer Fein*struktur und Zahl nicht von gesunden Kontrollen; saure Phosphatase, ebenfalls ein Enzym primärer Granula, ist in normaler Konzentration vorhanden. Hingegen fällt die Sudanschwarz-B-Reaktion, parallel zum

Schwester Grossmutter väterlicherseits) zeigten eine normale Granulozytenfunktion *in vitro*

*Immunologische Befunde*

Die *Lymphozyten* von W H während der Remission und vom Vater waren nach Zahl und Morphologie normal Die quantitative Bestimmung der T Lymphozyten im peripheren Blut ergab für Patient (56%) und Vater (61%) ebenso Normalwerte, wie die Bestimmung der B-Lymphozyten (Patient 21%, Vater 18%) *Serumimmunglobuline* bei W H lagen im Normbereich IgG 1190 mg%, IgA 186 mg%, IgM 90 mg%, IgE war mit 156 U/ml grenzwertig erhöht

*Komplement* Wiederholte quantitative Bestimmungen der Komplementkomponenten C3 C4 und C5 sowie der hämolytischen Aktivität CH50 ergaben für den Patienten, seine beiden Eltern und seine Schwester Werte im Normbereich

## *Diskussion*

*MPO* ist ein kationisches lysosomales Enzym der neutrophilen Granulozyten und der Monozyten Im feinstrukturellen Bereich lässt sich das Enzym zunächst im endoplasmatischen Retikulum, im Golgi Apparat und in den primären (azurophilen) Granula der Promyelozyten lokalisieren. Im Myelozyten und reifen neutrophilen Granulozyten ist es sodann nur noch in den primären Granula beim Monozyten in einem Teil der Monozytengranula festzustellen [3 5] Eosinophilen Peroxidase unterscheidet sich chemisch von MPO [2] Die physiologische Bedeutung der MPO und ihres Substrates H O wird in einer Mithilfe bei der Abtötung phagozytierter Mikroorganismen gesehen [Übersicht 25]

Die wenigen bisher beobachteten Beispiele eines *konstitutionellen MPO-Defektes* [16 23 32] bestätigten eine gemeinsame genetische Kontrolle dieses Enzyms für die Neutrophilen und Monozytenreihe nicht jedoch für die Eosinophilen. Entsprechend war auch bei den von uns untersuchten Fällen lediglich die Aktivität der MPO eingeschränkt. Familienuntersuchungen lassen einen autosomal rezessiven Erbgang vermuten 4 Söhne eines Patienten mit vollständigem Fehlen der MPO [16] zeigten eine deutlich verminderte Peroxydaseaktivität ihrer Neutrophilen. Die von uns untersuchte Familie lässt eine Heredität lediglich vermuten Beim klinisch gesunden Vater war die MPO-Aktivität in etwa 50% der Neutrophilen fehlend oder deutlich herabgesetzt beim an einer Leukämie

dem waren keine hämatologischen Besonderheiten nachzuweisen. Die Feinstruktur der Neutrophilen und Monozyten des Patienten war unauffällig; beim elektronenmikroskopischen Peroxidasenachweis blieben ihre Granula ungefärbt. Beim Vater mit partiellem Peroxidasedefekt fanden sich fließende Übergänge zwischen Neutrophilen mit positiven und solchen mit negativen primären Granula. Die intrazelluläre Abtötung phagozytierter Bakterien war beim Patienten stark, beim Vater deutlich eingeschränkt. Normal waren: NBT-Test, chemotaktische Leistung der Granulozyten, serumabhängige Phagozytose, Lymphozytenzahl und Anteil der B- und T-Lymphozyten, Serum-Immunoglobuline und mehrere Komplementkomponenten. Der Zusammenhang zwischen Peroxidasedefekt und Leukämie wird diskutiert, es wird eine Übersicht über bisher publizierte Fälle von Myeloperoxidasedefekt gegeben.

## *Dank*

Für die zuverlässige Durchführung der zytochemischen und elektronenmikroskopischen Arbeiten danken wir Frl. M. Darsow und Frl. G. Wochinger, der immunologischen Untersuchungen Frl. L. Jeppesen.

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Peroxidasedefekt, negativ aus. Dies ist eine weitere Bestätigung für die Annahme, dass durch diese Reaktion in den Neutrophilen ebenfalls Peroxidaseaktivität nachgewiesen wird [27] nicht jedoch Ablagerungen von Lipiden Beim partiellen MPO-Defekt des Vaters fanden sich sowohl licht wie elektronenmikroskopisch nebeneinander Neutrophile mit fehlender mit verminderter sowie mit normaler Peroxidaseaktivität Desgleichen fehlte das Enzym in einem Teil der Monozyten In Übereinstimmung mit diesem Befund war das *in vitro* getestete Bakterienabtötungsvermögen der isolierten Granulozyten immer deutlich schlechter als bei einer vergleichbaren Kontrollperson

Bei *Leukämiepatienten* ist ein partieller MPO-Defekt nicht selten Bei 12 von 28 Patienten mit unreifzelliger myeloischer Leukämie fehlte das Enzym in 8–70% der Blutneutrophilen [9] bei einem weiteren Patienten sogar in 95% der Neutrophilen [11] Bei Leukämiepatienten kann der MPO-Defekt in seltenen Fällen auch auf einem vollständigen Fehlen primärer Granula beruhen [4] Weiterhin sehen wir bei unreifzelligen myeloischen Leukämien nicht selten Defekte weiterer Enzymsysteme der myeloischen Zellreihe sowie morphologische Anomalien der Leukozytengranula [29] Sofern funktionelle Untersuchungen durchgeführt wurden, liess sich auch bei diesen Leukämiepatienten eine Korrelation zwischen verminderter Peroxidaseaktivität und *(in vitro)* gestörter Bakterienabtötung feststellen [10 31] Zytochemische Untersuchungen der Blutzellen können also in derartigen Fällen möglicherweise eine besondere Infektgefährdung einer bestimmten Patientengruppe aufdecken Sofern bei Leukämiepatienten zytochemisch normale und defekte Leukozyten nebeneinander auftreten so bleibt zu diskutieren ob es sich bei ersteren um Zellen der normalen Resthämatopoese, bei letzteren um defekt ausgereifte Zellen des leukämischen Zellklons handelt

In seltenen Fällen wurde ein wechselnder Anteil MPO-negativer Neutrophiler bei Patienten mit refraktärer Anämie beobachtet [1 8 12, 17] Bei diesen Patienten stützt der Enzymdefekt den Verdacht auf das Vorliegen einer *Präleukämie* [4]

## *Zusammenfassung*

Bei einem 15jährigen Patienten mit akuter myeloischer Leukämie wurde während der akuten Phase der Erkrankung und in Remission ein vollständiger Myeloperoxidasedefekt der Neutrophilen und der Monozyten festgestellt Beim Vater des Patienten bestand ein partieller Defekt dieses Enzyms, bei weiteren Familienmitglie-

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_5 SBARRA, A. J PAUL, B STRAUSS, R and MITCHELL, G W jr Metabolic and

In the present study we compared the phagocytic activity of PMNs after capping of their ricin binding sites with that of unperturbed PMNs. We have used ferritin-labelled ricin in order to be able to follow the fate of the lectin, and have evaluated phagocytosis and the concomitant membrane redistribution by means of ultrastructural morphometry.

Our results indicate that in human PMNs, ricin-binding sites are not involved in the process of recognition and uptake of two test particles, baker's yeast and *Staphylococcus epidermidis*. This confirms and extends our preliminary observations on the effects of concanavalin A and ricin on the phagocytic activity of rabbit PMNs [9]. A preliminary communication on this work has already appeared [3].

## *Materials and Methods*

*Polymorphonuclear leukocytes.* Human peripheral blood obtained from healthy volunteers was anticoagulated with ACD solution [6], immediately diluted with one fifth of its volume with a solution containing 6 g of dextran T 500 in saline, and allowed to sediment in a graduated cylinder at room temperature. The white cell layer was then collected by aspiration, centrifuged at 5,500 g-min at 4 °C, and the PMNs were partially purified by washing the cell pellet three times in saline (300 g-min at 4 °C). The final cell suspension, which consisted of nearly 80% PMNs, was made up in a medium containing 12. mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, and 16.7 mM sodium cacodylate-HCl buffer, pH 7.4 (medium A).

*Lectin preparation.* A ferritin conjugate of ricin (RF) was prepared within 10 days of use, according to a modification of the method of DE PETRIS and RAFF [5], and stored at 4 °C in 0.1 M sodium cacodylate buffer, pH 7.4. The RF stock solution used in the experiments presented here had a protein content of 270 µg/ml. The ricin to ferritin ratio was 1.7:1. The RF concentrations used (16.9, 67 and 135 µg/ml) correspond to native ricin concentrations of 7.4, 29.3 and 59.2 µg/ml, respectively. Occasional clumps were eliminated by centrifuging the RF preparation at 12,000 rpm for 5 min in a Beckman microfuge immediately before use (Beckman Instruments, Palo Alto, Calif.).

*Lectin treatment of PMN.* Appropriate dilutions of the RF solution were added to 0.5 ml of the cell suspension ($12 \times 10^6$ PMN/ml) and kept in an ice bath for 10 min with periodic agitation. 3 vol of ice-cold medium A were then added, and the cells were cleared of excess lectin by low-speed centrifugation (1,000 g-min) at 4 °C. The cells were then resuspended in 1 ml of cold medium A and placed for 10 min in a 37 °C water bath in order to induce the formation of caps. Control cells were handled identically omitting the lectin solution which was replaced by an equal volume of the corresponding buffer. The lectin treatment induced some cell aggregation. The cells, however, were easily dispersed during resuspension.

*Phagocytosis.* Two test particles, baker's yeast and *S. epidermidis*, were used. Fresh baker's yeast was suspended in saline (3.2 g in 7 ml), heated for 10 min in

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# Capping of Ricin Binding Sites Does Not Influence Phagocytosis in Human Polymorphonuclear Leukocytes

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*Key Words* Ricin Capping PMN Phagocytosis Morphometry

*Abstract* Human polymorphonuclear leukocytes (PMNs) were incubated at 0 C with ferritin-conjugated ricin and warmed to 37 C to induce capping of the ricin-binding sites. The PMNs were then allowed to phagocytose yeast or *Staphylococcus epidermidis* for 15 min, and processed for electron microscopy Phagocytic uptake, granule fusion, and the fate of lectin-bound membrane were quantified by morphometry Ricin-capped PMNs phagocytosed as extensively as untreated PMNs. Particles were ingested almost exclusively with a lectin-free portion of the plasma lemma. Fusion of granules with phagocytic vacuoles was not affected by ricin-induced capping. This indicates that ricin-binding sites are not involved in particle recognition and uptake.

## Introduction

When added to resting polymorphonuclear leukocytes (PMNs), concanavalin A [20] or ricin [DEWALD unpubl.] induce a metabolic burst similar to that which accompanies phagocytosis. The metabolic response appears to result from the specific interaction of the lectins with glycosidic residues on the PMN surface since it is prevented by the presence or terminated by the addition of the respective sugar haptens, α-methyl mannoside and α-lactose These observations suggested that certain lectin binding sites on the PMN plasma membrane may participate in the recognition of particles to be phagocytosed and seemed in accordance with a report by BERLIN [4] claiming that concanavalin A inhibits phagocytosis in PMNs.

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higher magnification. This stratified sampling technique [24] was adopted in order to ensure that each cell was evaluated only once. The electron micrographs were taken on Zeiss EM 9-S microscope (Carl Zeiss, Oberkochen, FRG) at original magnifications of 1,870 and 5,104 and were calibrated using carbon-grating replica containing 2,160 lines/mm. Morphometry was performed on negatives which ere enlarged 4.8 times by projection out the ppropriate counting lattice.

*Morphometry surface and olum densities.* Surface density of lectin-tagged and lectin-free membrane was estimated using the technique outlined by WEIBEL [24]. This procedure involves counting the intersections between cellular membranes and superimposed lattice. A curvilinear test lattice [15] as used to eliminate the counting error introduced by the inherent anisotropy of capped surfaces. Membrane was considered as lectin-bound if, t the point of intersection with the test system, ferritin molecule was within 20 nm from the membrane [8]. The intersection counts thus obtained are directly related to surfac density. Data are expressed as relative surface density: that is, as fraction of the total cellular surface density. Relative olume density of the phagosomal compartment was determined using the point counting method [24].

*Calculation and statistics.* All calculations of surface, and volume densities were performed for each PMN individually and then eraged for each experimental condition. A two-tailed significance matrix was constructed for each parameter hich allowed statistical comparison of each mean value against every other mean value within each experimental group. All calculations were performed using Hewlett-Packard 2100 S computer.

## *Results*

### *Lectin-Induced Capping*

When human PMNs were exposed to RF at a temperature near 0 C, the lectin bound uniformly to the cell surface. Sections of such PMNs showed a continuous layer of electron-dense ferritin dots on the plasmalemma (fig. 1). When the PMNs, after elimination of unbound RF were warmed to 37 C the lectin collected at one pole of the cell to form a cap (fig. 1 inset; fig. 3–6). This process was completed within 10 min, as judged by the absence of uniformly RF-labelled cells, and by the very rare occurrence of RF patches. Lectin-induced capping has been observed in PMNs by RYAN *et al.* [22] using fluorescence microscopy. The more detailed view afforded by electron microscopy shows that the cap is generally sharply delineated, and that the plasma membrane bearing the cap is often infolded (fig. 4, 5). Cap formation in human PMNs occurred as rapidly and as regularly as in rabbit PMNs [9] and was not affected by the concentration of RF to which the PMNs were exposed. The size of the cap was not significantly different in PMNs which were treated with 16.9

boiling water bath, washed by centrifugation, and resuspended in 10 ml of saline. *S. epidermidis* (ATCC 12,228) was grown for 24–48 h in brain-heart infusion broth (BHI, Difco Laboratories, Detroit, Mich.), collected by centrifugation, resuspended in saline to a density of $3.3 \times 10^9$ cells/ml and killed by heating for 30 min at 60 °C. The bacteria were then centrifuged at approximately 30,000 g-min, and resuspended in one-sixth of the original volume of saline. Immediately before use, each particle suspension was mixed with an equal volume of fresh human serum, and opsonized for 30 min at 37 °C. Phagocytosis was started by adding 50 µl of either suspension of opsonized particles to $6 \times 10^6$ of lectin-treated or untreated PMNs in 1 ml of medium A. Particle to PMN ratios were 50:1 for yeast and 80:1 for staphylococci. Final serum concentration was 2.5% (v/v). Phagocytosis was stopped after 15 min by the addition of an equal volume of ice-cold 3% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The samples were kept in the fixative at 4 °C for 60 min. Small pellets were then prepared by centrifuging 400 µl clump-free portions (see 'Discussion') of the fixed cell suspension for 5 min at 12,000 rpm in a Beckman microfuge. The pellets were removed from the tubes and left overnight at 4 °C in 0.1 M sodium cacodylate buffer, pH 7.4, containing 5% (w/v) sucrose.

*Biochemical phagocytosis assay.* In some cases, phagocytosis was assayed by determining the radioactivity associated with the PMNs after incubation with radioactively labelled bacteria. *S. epidermidis* (ATCC 12,228) was grown in 100 ml of BHI broth in the presence of 0.1 mCi of uniformly-labelled $^{14}$C glucose (NEN Chemicals GmbH, Frankfurt, FRG), and then prepared for use as described for the unlabelled bacteria. Phagocytosis was started by adding 50 µl of opsonized staphylococci (see above) to $6 \times 10^6$ PMNs suspended in 0.5 ml of medium A, and stopped after 15 min by adding 2.5 ml of ice-cold saline and transferring the samples to an ice bath. The PMNs were separated from the bacteria by centrifuging at 700 g-min at 4 °C and washing twice with 3 ml of saline under the same conditions. The washed PMN pellets were solubilized in 1 ml of 0.5% (v/v) triton X 100, mixed with 12 ml of a 12% (v/v) solution of ethanolamine in methanol and 9 ml of 0.6% (w/v) solution of 2-(4-tertiary butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadizol (PBD, Fluka AG, Buchs, Switzerland) in toluene and counted in a Beckman LS 133 scintillation counter (Beckman Instruments). Blank samples were treated in exactly the same way but were kept in ice during the phagocytosis period.

*Cytochemistry.* After overnight washing in buffer, all glutaraldehyde-fixed samples were incubated for 2 h in the medium of GRAHAM and KARNOVSKY [10] for the cytochemical demonstration of peroxidase, which is an ultrastructural marker for azurophil granules [2].

*Electron microscopy.* The pellets were stained en bloc with magnesium uranyl acetate and embedded according to standard procedures [14]. From each experimental situation, three randomly-chosen blocks were sectioned. After several silver sections were placed on a grid, a 10-µm section was sliced off the block and cutting was resumed. This process was repeated 5–6 times. The sections were stained on grid for 20 min with lead citrate. On each grid, only the cells included in a single grid space were evaluated. The field was first photographed at low magnification, and cell profiles containing at least one particle were photographed individually at

*Table I* Percent of PMN population phagocytosing

| Additions to PMNs RF µg/ml | particle | Percent of PMN profiles containing test particles (mean ± SEM) | Number of grids examined |
|---|---|---|---|
| 0 | yeast | 9 3 ± 8 | 16 |
| 16.9 | yeast | 90 7 ± 2 7 | 17 |
| 67.0 | yeast | 93 1 ± 2 5 | 12 |
| 135.0 | yeast | 88 0 ± 2 3 | 15 |
| 0 | *S. epidermidis* | 86 8 ± _9 | 13 |
| 16 9 | *S epidermidis* | 89.2 ± 2.4 | 15 |
| 67.0 | *S epidermidis* | 89 4 ± 2 5 | 14 |
| 135.0 | *S epidermidis* | 85 3 ± 2 7 | 16 |

N statistically significant difference between any pair of values at confidence level of 95%.

One grid space per grid was photographed ( 1,870) according to the stratified sampling procedure (see Methods)

*Phagocytosis*

We have compared the phagocytic activity of RF-capped PMNs with that of untreated cells. After confirming the data obtained with rabbit PMNs [9] in a few preliminary experiments using both test particles, we performed one experiment incorporating all the conditions tested, which was then evaluated by morphometry Since phagocytosis was quantified only on PMN profiles containing particles the incidence of such cells under the various experimental conditions was assessed on low-magnification micrographs (fig 2) Table I shows that particles were present in 85–93* of the PMNs viewed. None of these percent values is statistically different from any other at a confidence level of 95 *, thus indicating that under all experimental conditions about the same proportion of PMNs had phagocytosed. Figure 2 is an example of low magnification micrographs which were used for the above counting. Capped PMNs which have phagocytosed either yeast or staphylococci are shown in figures 3 and 4 The caps (dotted lines) are seen as a multiple layer of ferritin dots covering an appreciable segment of the plasmalemma (fig. 5 6). Occasional ferritin-loaded vesicles are seen near the cap (fig. 3–6). They reflect either endocytosis or deep invaginations (fig 4) of the cap.



*Fig. 1.* Detail of a PMN which was treated with RF (16.9 μg/ml) and fixed at 0 °C. RF is seen as a thin uniform layer of electron-dense dots on the cell surface. Staining for peroxidase activity allows a clear differentiation between azurophil (A) and specific (S) granules. × 20,000. Inset: Detail of a PMN which was treated with RF as above and then allowed to cap by incubation at 37 °C before fixation. As compared to the above case, the cap appears as a thicker and more tightly packed layer of RF. The arrow indicates the sharp boundary of the cap. Specimen preparation as in figure 2. Bar equals 1 μm.

67 or 135 μg RF/ml (data are given in table III). This observation and the fact that large amounts of free lectin were recovered after washing, suggest that RF was used in excess, and that virtually all RF-binding sites were covered. By analogy to what is thought to occur in lectin-treated lymphocytes [16], we expect RF to move into the cap together with the plasma membrane molecules to which it binds, thus depleting the remainder of the plasma membrane of RF binding sites. Transition to 37 °C did not seem to uncover additional RF-binding sites, since no difference in cap size was observed when PMNs were warmed to 37 °C in the presence of excess RF. In a number of PMNs, occasional ferritin molecules were detected outside the cap area. This phenomenon, which has also been observed in lymphocytes [18], may indicate that a few RF binding sites are left behind at random during cap formation. Alternatively, these lectin molecules may be bound to a minor population of less mobile sites. Under the experimental conditions adopted, no RF-binding to the surface of opsonized baker's yeast or *S. epidermidis* was observed.

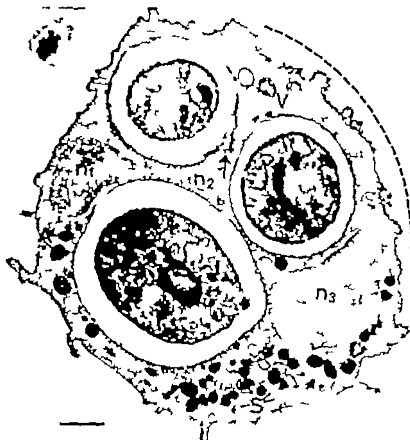



*Fig 3* RF-capped PMN which has ingested yeast RF is packed in the sharply delineated cap area (dotted line) and absent on the remaining portion of the plasmalemma. The membranes of the three phagocytic vacuoles are completely free of RF indicating that only membrane which had been depleted of ricin-binding sites was involved in the ingestion process T RF-containing vesicles (V) are seen in the vicinity of the cap They may indicate cap uptake or deep invaginations of the RF bound surface Peroxidase-positive azurophil (A) and peroxidase-negative specific (S) granules are seen. A possible sit of fusion of granule with phagocytic vacuole is indicated (arrow). $-n_1$ = nucleus. This preparation was treated with 67 g RF/ml. For details, see Methods Specimen preparation as in figure 2. Bar equals 1 μm.

buffer dehydrated in ethanol (70-100%), passed through propylene oxide, and embedded in Epon Sections were stained for 20 min with lead citrate Bar equals 1 μm

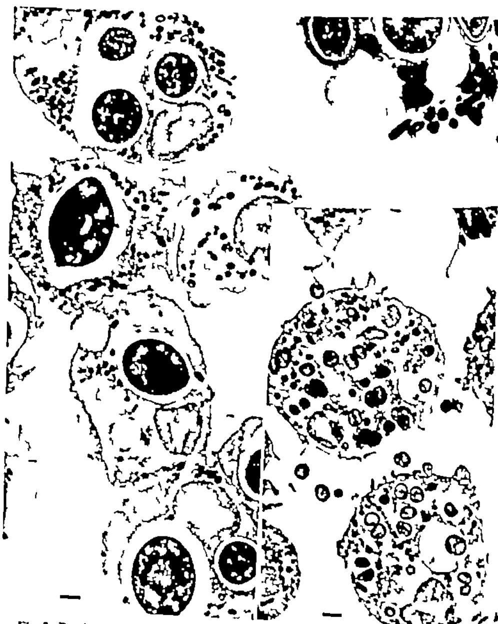


*Fig 2* Portions of survey micrographs of RF-capped PMNs which have been exposed to yeast (a) or *S epid rmidis* (b) RF cannot be seen at this magnification. Note the eosinophilic leukocyte (upper right) which has engulfed three yeast particles and exhibits extensive reaction product for peroxidase within the phagosomes. These cell suspensions were treated with 16.9 g RF/ml For incubation cond'tions, see Methods Specimens were fixed in 1.5% glutaraldehyde in 0 1 M sodium cacodylate buffer pH 7 4 for 1 h at 4 C washed overnight in 0.1 M sodium cacodylate buffer pH 7 4 containing 5% sucrose incubated in the peroxidase med um of GRAHAM and KARNOVSKY [10] for 2 h t 22 C, refixed 1 1 / OsO in 0 1 M cacodylate

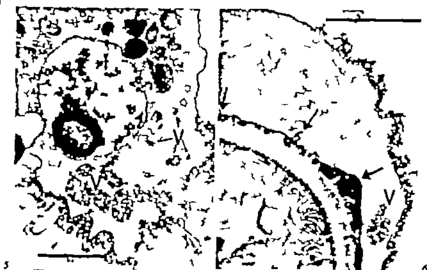


*Fig 5* Detail of capped PMN which has ingested *S epidermidis* Peroxidase reaction product is present in the phagosome, the membrane of which is free of apposed RF A substantial portion of the cap has been internalized as suggested by the formation of lectin-filled vesicles (V) For incubation conditions, see Methods This cell was treated with 135 µg RF/ml Specimen preparation as in figure 2. Bar equals 1 m

*Fig 6.* Higher magnification of the cap ut of PMN which has ingested yeast. Except for single RF-filled vesicle (V), the entire cap has remained on the exterior of the cell. Arrow indicate extensive fusion of azurophil granules with the phagosome. For incubation conditions, see Methods' This cell was treated with 67 µg RF/ml. Specimen preparation as in figure 2 Bar equals 1 µm.

were supplied with opsonized yeast, the mean relative surface density of the phagosome compartment (number of intersections between counting lattice and phagosome membrane as percent of total membrane intersections in the PMN) was between 17 7 and 22.9 This figure was somewhat lower (11.6–16.5) in PMNs that had phagocytosed staphylococci. As expected, in view of the very different size of the two test particles, the relative volume density of the phagosome compartment (reflecting the per cent of PMN volume occupied by phagosomes) is much larger in PMNs which have ingested yeast (37 6–45 7) than in those which have ingested staphylococci (15 0–15.5). More important than the actual values is the fact that in *no case* were the average surface or volume densities of the




*Fig. 4.* An RF-capped PMN which has ingested *S. epidermidis.* The cap (dotted line) induced with 16.9 μg RF/ml, shows a deep invagination. Three RF-filled vesicles can be seen in the vicinity of the cap site. Reaction product for peroxidase is present to various degrees in the phagosomes, all of which must have originated from RF free plasma membrane. One microorganism apparently is attached to a portion of the plasma membrane near the cap boundary. For incubation details, see Methods. Specimen preparation as in figure 2. A = azurophil granule. S = specific granule. n = nucleus. Bar equals 1 μm.

We have determined relative surface and volume densities of the phagosome compartment as a measure of phagocytic activity. These two sets of data are obtained separately by intersection and point counting, respectively, and therefore represent independent measurements of the same phenomenon. These data are presented in table II. In PMNs that

*Table III* Lectin distribution in capped PMN

| Additions to PMNs RF µg/ml | particle | Percent of RF-bound membrane PMN surface $\frac{S}{S_{PMT}}$ 100 | Phagosome inner surface $\frac{S_{PG}}{S_{POT}}$ 100 |
|---|---|---|---|
| 16 9 | none | 19 9 ± 1 7 | |
| 16 9 | yeast | 15 7 ± 2 2 | 1 6 ± 1.0 |
| 67 0 | yeast | 18 9 ± 1 4 | 0.4 ± 0.3 |
| 135.0 | yeast | 19 9 ± 0 | 1.0 ± 0 6 |
| 16 9 | *S epidermidis* | 15 1 ± 1.4 | 2.0 ± 1 0 |
| 67.0 | *S epidermidis* | 17.5 ± 2 0 | 2.8 ± 1 1 |
| 135.0 | *S. epidermidis* | 1 0 ± 1 | 7.6 ± [illegible] |

*Mean values ± SEM from 20 single PMN for each experimental condition.* Data represent surface densities (S ) of RF-bound plasma membrane (PMB) or RF-bound phagosome membrane (PGB) as percent of total plasma or phagosome membrane (PMT or PGT).

*Table IV* Morphometric evaluation of granule fusion in phagocytosing PMNs

| Additions to PMNs RF µg/ml | particle | Specific granules $\frac{S_{V\,SP}}{S_{V\,TOT}}$ 100 | Azurophil granules $\frac{S_{AZ}}{S_{V\,TOT}}$ 100 |
|---|---|---|---|
| 0 | none | 39 7 ± 1 | 38 7 ± 1.2 |
| 16.9 | none | 38 6 ± 1 1 | 41.8 ± 1.3 |
| 0 | yeast | 15.7 ± 1 2 | 26.8 ± 1 7 |
| 16.9 | yeast | 15.9 ± 1 5 | 23.5 ± 1 9 |
| 67.0 | yeast | 15 7 ± 1 1 | 22 8 ± 1.5 |
| 135.0 | yeast | 15 9 ± 1 6 | 27.2 ± 2.1 |
| 0 | *S epidermidis* | 20 4 ± 1 4 | 31.5 ± 1 4 |
| 16 9 | *S epidermidis* | 18 9 ± 1 9 | 29.5 ± 1.8 |
| 67 0 | *S epidermidis* | 19 4 ± 1.2 | 30.3 ± 2 0 |
| 135.0 | *S epidermidis* | 19 4 ± 1.2 | 31.9 ± 1.9 |

Surface density (S ) of specific (SP) and azurophil (AZ) granule membrane are expressed as a percent of the total (TOT) cell surface density

Mean values ± SEM from 40 PMNs. All other values were obtained from 20 PMNs.

*Table II.* Morphometric evaluation of phagocytosis: relative surface and volume densities of phagosome space[1]

| Additions to PMNs RF, μg/ml | particle | $\frac{Sv_{PG}}{Sv_{TOT}} \times 100$ | $\frac{Vv_{PG}}{Vv_{TOT}} \times 100$ |
|---|---|---|---|
| 0 | yeast[2] | 19.5 ± 1.3 | 41.3 ± [illegible] |
| 16.9 | yeast | 22.9 ± 1.6 | 44.0 ± [illegible]5 |
| 67.0 | yeast | 2[illegible].0 ± 1.8 | 45.7 ± 3.4 |
| 135.0 | yeast | 17.5 ± [illegible] | 37.6 ± 3.7 |
| 0 | *S. epidermidis*[2] | 14.4 ± 1.1 | 15.5 ± 1.4 |
| 16.9 | *S. epidermidis* | 15.4 ± [illegible]1 | 15.0 ± 1.7 |
| 67.0 | *S. epidermidis* | 11.6 ± 1.7 | 15.1 ± [illegible]4 |
| 135.0 | *S. epidermidis* | 13.5 ± 1.8 | 15.0 ± [illegible]6 |

[1] Surface density (Sv) and volume density (Vv) of phagosome space (PG) are expressed as percent of the total cell surface and total cell volume densities, respectively.

[2] Mean values ± SEM from 40 single PMNs. All other mean values were obtained from 20 PMNs.

phagosome compartment of RF-capped PMNs significantly different from those of control PMNs. These measurements of phagocytic activity therefore clearly indicate that induction of an RF cap does not affect the ability of PMNs to ingest opsonized yeast or staphylococci.

*Lectin Distribution in Phagocytosing PMNs*

By means of the ferritin tag, we were able to observe the localization and to follow the redistribution of ricin during phagocytosis. As shown in table III, at the end of the experiment the cap occupied between 15.1 and 21% of the PMN surface. There was no significant difference in cap size of phagocytosing and resting PMNs. Moreover, cap size was not a function of the amount of RF to which the PMNs were originally exposed. By contrast, the proportion of phagosome membrane associated with lectin was very small after ingestion of yeast (0.4–1.6%) and in 2 of 3 cases after ingestion of staphylococci (2 and 2.8%). Even in the last experimental case (PMNs capped at the highest RF concentration and exposed to staphylococci), the percent of phagosome membrane bearing the lectin tag was much smaller (about one third) than the percent of plasma lemma occupied by the cap. The absence of lectin from the phagosome

*Table III* Lectin distribution in capped PMNs

| Additions to PMNs | | Percent of RF-bound membrane | |
|---|---|---|---|
| RF $\mu$g/ml | particle | PMN surface $\frac{S_{V_{PM}}}{S_{V_{PMT}}} \cdot 100$ | Phagosome inner surface $\frac{S_{V_{PG}}}{S_{V_{PGT}}} \cdot 100$ |
| 16.9 | none | 19.9 ± 1.7 | |
| 16.9 | yeast | 15.7 ± [illegible] | 1.6 ± 1.0 |
| 67.0 | yeast | 18.9 ± 1.4 | 0.4 ± 0.3 |
| 135.0 | yeast | 19.9 ± [illegible] | 1.0 ± 0.6 |
| 16.9 | *S epidermidis* | 15.1 ± 1.4 | 2.0 ± 1.0 |
| 67.0 | *S epidermidis* | 17.3 ± 2.0 | 2.8 ± 1.1 |
| 135.0 | *S epidermidis* | 21.0 ± 2.1 | 7.6 ± 2.[illegible] |

Mean values ± SEM from 20 single PMNs for each experimental condition. Data represent surface densities ($S_V$) of RF-bound plasma membrane (PMB) or RF-bound phagosome membrane (PGB) as percent of total plasma or phagosome membrane (PMT or PGT)

*Table IV* Morphometric evaluation of granule fusion in phagocytosing PMNs

| Additions to PMNs | | Specific granules | Azurophil granules |
|---|---|---|---|
| RF $\mu$g/ml | particle | $\frac{S_V}{S_{V_{TOT}}} \cdot 100$ | $\frac{S_{V_{AZ}}}{S_{V_{TOT}}} \cdot 100$ |
| 0 | none | 39.7 ± 1.2 | 38.7 ± 1.[illegible] |
| 16.9 | none | 38.6 ± 1.1 | 41.8 ± 1.3 |
| 0 | yeast | 15.7 ± 1.2 | 26.8 ± 1.7 |
| 16.9 | yeast | 15.9 ± 1.5 | 23.5 ± 1.9 |
| 67.0 | yeast | 15.7 ± 1.1 | 22.8 ± 1.5 |
| 135.0 | yeast | 15.9 ± 1.6 | 27.2 ± 2.1 |
| 0 | *S epidermidis*[a] | 20.4 ± 1.4 | 31.5 ± 1.4 |
| 16.9 | *S epidermidis* | 18.9 ± 1.9 | 29.5 ± 1.8 |
| 67.0 | *S epidermidis* | 19.4 ± 1.2 | 30.3 ± 2.0 |
| 135.0 | *S epidermidis* | 19.4 ± 1.2 | 31.9 ± 1.9 |

Surface density ($S_V$) of specific (SP) and azurophil (AZ) granule membrane are expressed as a percent of the total (TOT) cell surface density

Mean values ± SEM from 40 PMNs. All other values were obtained from 20 PMNs.

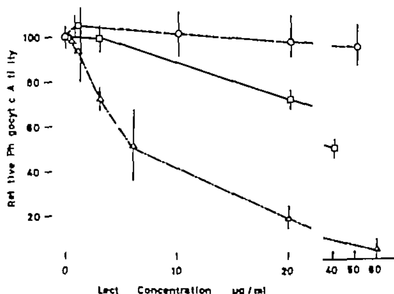



*Fig 7* Effects of lectins on phagocytosis of [14]C labelled *S epidermidis* by human PMNs. The PMNs were pretreated in the cold with various concentrations of concanavalin A (O), RF (□) or native ricin (△), cleared of excess lectin, transferred to 37 C for cap induction, and then exposed to labelled bacteria for 15 min. Graphs show relative amount of radioactivity associated with the PMNs as a function of the concentration of the lectins used in the pretreatment. Vertical bars represent the range of triplicate values. For details, see 'Methods'

membrane can be best observed in figures 3 and 5 These results indicate that both test particles were taken up almost exclusively by lectin free portions of the plasma membrane

*Fusion of Granules with the Phagosome*

The presence of peroxidase reaction product in the phagocytic vacuoles (fig 3–6) indicates that in RF-treated PMNs fusion of azurophil granules had occurred. We have quantified granule fusion by calculating the relative surface density of intact azurophil and specific granules. As shown in table IV each type of granule contributes approximately 39% of the total cell membrane. Capping PMNs with RF did not significantly change these figures. As expected however granule surface dropped drastically upon phagocytosis. In PMNs which phagocytosed yeast specific and azurophil granule membrane were down to about one third and

two-thirds, respectively of the resting controls within 15 min. A drop of about 50% for specifics, and ~5 for azurophils was observed after phagocytosis of staphylococci Pretreatment of the PMNs with RF did not influence the disappearance of either type of granule, since very similar data were obtained within each experimental condition in which particles were provided.

*Radiochemical Measurement of Phagocytosis*

The morphometrical studies were complemented by a small series of experiments in which phagocytosis was tested in suspensions of PMNs exposed to radio-labelled *S epidermidis* and uptake was expressed as the amount of PMN-associated radioactivity Under such conditions, the study of specific lectin effects may be disturbed by lectin-induced PMN aggregation which partially hinders the free approach between PMNs and particles, thus precluding a valid comparison between lectin-treated and control PMNs (see Discussion). The results obtained are shown in figure 7 Pretreatment of the PMNs either with ricin or its ferritin conjugate resulted in an apparent decrease in overall particle uptake. Native ricin was considerably more inhibitory than RF By contrast, pretreatment with concanavalin A up to a concentration of 40 μg/ml did not influence uptake. Uptake inhibition appears to correlate with the lectin's capacity to aggregate PMNs and is probably a consequence of the restricted access of particles to the clumped PMNs. In the case of native ricin, a cytotoxic effect seems likely since over 50% of the total PMN LDH was found to be released.

## Discussion

Using stereological techniques, we have studied the effect of surface alteration by ricin-induced capping on the phagocytic activity of human PMNs. PMNs which were capped with ferritin-labelled ricin over a wide concentration range phagocytosed either opsonized yeast or opsonized *S epidermidis* as effectively as nontreated PMNs. Azurophil and specific granules fused rapidly and extensively with the phagocytic vacuoles formed RF-capping did not affect the extent of fusion of either type of granule RF-bound membrane was found only rarely in particle-containing vacuoles.

These observations lead to the following conclusions (1) ricin-binding

sites appear not to be necessary for particle recognition and uptake by human PMNs (2) in RF-capped PMNs only that portion of the plasmalemma which has been depleted of ricin binding sites participates in particle ingestion (3) phagocytic vacuoles formed from plasmalemma which has been depleted of its ricin-binding sites fully retain the structural properties required for fusion with azurophil and specific granules.

In an earlier study we obtained identical results using rabbit peritoneal exudate PMNs which were capped with either RF or a ferritin conjugate of concanavalin A and then allowed to phagocytose yeast [9]

It would seem that ricin and concanavalin A induce a metabolic burst in PMNs by nonspecifically perturbing the plasma membrane rather than by interacting with particle recognition sites. Nonspecific membrane perturbation leading to an increase in cyanide-insensitive oxygen consumption can be induced by disparate agents like phospholipase C, surfactants, and fatty acids [21]

Our findings are in apparent contrast with those of BERLIN [4] who observed an inhibition of phagocytosis of polyvinyltoluene beads in adherent rabbit PMNs during exposure of concanavalin A The different experimental conditions adopted preclude a comparison of these data with ours. It is possible, however that in BERLIN's experiments phagocytosis was inhibited by a nonspecific hindrance of particle attachment rather than by blockade of particle recognition sites, since concanavalin A and the particles were added simultaneously to the PMNs. Indeed such a possibility was considered in that study In our hands, prior capping of concanavalin A binding sites had no inhibitory effect on either rabbit PMNs phagocytosing yeast [9] or on human PMNs phagocytosing radioactively labelled *S epidermidis* (fig 7) Similarly EDELSON and COHN [7] found that concanavalin A had no effect on the ability of adherent mouse macrophages to ingest IgG-coated sheep red blood cells or latex particles. The involvement of lectin-binding sites in particle uptake by PMNs has also been suggested by a study showing a diminished concanavalin A and ricin-binding capacity of plasma membrane isolated from PMNs which had phagocytosed [17] The conclusions of this paper are based on the assumptions that the method used provides membrane preparations qualitatively and quantitatively representative of the plasmalemma of the PMNs studied and that comparable plasmalemmal preparations can be obtained from resting and phagocytosing PMNs. In view of the opposite evidence which we obtained directly by quantitative microscopy and of the great difficulties in preparing clean membrane fractions from PMNs [1 3] and

other cells [19] a much better biochemical characterization of the fractions obtained would seem an essential control of the above [17] study

A remarkable finding of this and the earlier work on rabbit PMNs [9] is the virtual exclusion of lectin-bound membrane from the phagosome. This phenomenon is particularly striking when test particles like yeast are used, which induce the internalization of large areas of the PMN plasmalemma. Formerly we interpreted these findings as indicating a polarization of the capped PMN [9] We reasoned that full exclusion of the cap could only occur when particles attach and are taken up at the opposite pole of the PMN This is in keeping with data given by RYAN *et al* [22] who observed concanavalin A caps at the trailing end of chemotactically attracted PMNs. The recent hypothesis (based on studies of mouse macrophages) of a zipper-like recognition mechanism between phagocyte membrane and particle [11 12] offers an interesting alternative explanation of our findings being covered by RF the cap area cannot attach to the test particle and therefore does not become part of the phagocytic vacuole Exclusion of RF-bound membrane from the phagocytic vacuoles may have resulted occasionally from attachment to the cap of another cell or cellular debris, thus hindering particle access to the cap area. However this could never result in the almost complete cap exclusion noted since most PMNs showed an unencumbered cap region. In PMNs which were capped at the highest concentration of RF and then allowed to phagocytose staphylococci, there was a somewhat higher incidence of RF-bound phagosome membrane than in all other cases. It is difficult to explain this finding since it does not occur when similarly treated cells ingest yeast, nor does it appear to be a function of cap size which, in this case, is slightly but nonsignificantly larger than caps induced with lower concentrations of RF Perhaps the uptake of bacteria is less orderly than that of the large yeast. The cells may have to polarize or *circumferentially* attach to ingest yeast, but bacteria attaching to the cell in the proximity of the cap (fig. 4) may passively include some RF-bound membrane. It is unlikely that lectin bound membrane in the phagosome could have been contributed by fusion with the small endocytic vacuoles originating from the cap. These RF-filled vesicles were never seen to fuse either with phagosomes or with granules (fig. 3–6). Similar observations were made by EDELSON and COHN [7] in mouse macrophages.

Table IV presents the first quantitative assessment of degranulation during phagocytosis, a phenomenon which cannot be measured biochemically We found no significant effect of the lectin on the disappearance of

either granule type Similarly in our earlier study on rabbit PMNs phagocytosing yeast, both RF and concanavalin A were without effect on degranulation [9] Fusion of both granules was more extensive after ingestion of yeast. This may simply indicate that in this case the likelihood of coalescence between granules and phagosomes was higher than upon uptake of staphylococci HOFFSTEIN *et al* [13] found a decrease in specific granule volume in resting rabbit PMNs which had been treated with high concentrations of native concanavalin A Under our experimental conditions treatment of resting human PMNs with RF did not significantly change the numbers of granules found in the cytoplasm.

*Methodological Considerations*

We have assessed phagocytic activity by measuring uptake 15 min after the addition of particles to the PMNs. We therefore lack information about the kinetics of the process However since particles ingestion in all cases was so extensive within the short time period, possible differences in uptake kinetics are unlikely to be relevant to our conclusions.

The clear-cut data obtained by morphometry do not correlate completely with measurements of overall uptake of radioactive bacteria by PMN suspensions. In these experiments, concanavalin A treatment was without effect but both ricin and RF were clearly inhibitory As stated in the results, PMN aggregation (which was strong after ricin or RF treatment) poses an insurmountable difficulty in this experimental setting. Biochemical determination of phagocytic activity by measurement of the total amount of cell associated particles assumes unobstructed access of cells to particles. Since lectins aggregate cells, this assumption is not justified and may lead to unwarranted conclusions regarding the effect of lectin treatment on phagocytic activity This problem and the well known difficulties in discriminating between adhering and engulfed particles [see 23 for discussion] are avoided in the morphometrical approach comparing individual cells which had ingested at least one particle and therefore can be assumed to have had equal access to the test particles. That the avoidance of obvious clumps during processing in lectin treated samples introduces no error is established by the results of table I control incubations, which showed no aggregation resulted in an equal percent of cells containing test particles A direct effect of RF on phagocytic performance (rather than indirect inhibition by agglutination) would have been observed as a diminution in the percent of unaggregated cells which had phagocytosed.

*Acknowledgments*

We thank Dr H. P. Schneebeli for his advice in preparing ricin-ferritin, and Ms. Verena Deller, Ms. Ursula Hornberger, and M. Bruno Streit for their technical assistance.

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# Oxacillin-Induced Granulocytopenia

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*Key Words.* Antileukocyte antibodies · Drug-induced granulocytopenia · Oxacillin · Penicillin

*Abstract.* The development of granulocytopenia during oxacillin therapy is reported in 2 patients. In both cases the onset of granulocytopenia occurred 24 days after therapy was instituted with similar clinical presentations. The bone marrow aspirates revealed myeloid hyperplasia, left shift and maturation arrest. 24 h after oxacillin was discontinued the granulocyte counts rapidly rose towards normal, with subsequent complete recovery. Although sought for, leukoagglutinins could not be demonstrated in either patient. The occurrence of granulocytopenia in patients receiving natural and semisynthetic penicillins is reviewed and the mechanisms are outlined.

Granulocytopenia following use of penicillin and its analogues has been reported infrequently [1–8]. Our hospital recently began using oxacillin and within the first 4 months two cases of oxacillin-induced granulocytopenia were observed. A detailed search of the current literature has failed to reveal previous reports of granulocytopenia involving this medication.

## *Case I*

An 18-year-old primigravida Black woman, in her 18th week of pregnancy, was admitted to the Boston City Hospital Medical Service with a 10-day history of sore throat, rhinitis and increasing cough. She had been treated with codeine and glycerol guaiacolate as an out-patient. On admission she was febrile (101 °F), tachycardic and tachypneic, with findings of consolidation at the right base. Admission labora-

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*Fig 1* Initial bone marrow (case No 1) shows normal myeloid maturation. Wright's Giemsa stain × 600

tory studies showed normal renal function, electrolytes and liver function. The hem atocrit was 31 / *with normal red blood cell indices and adequate platelets.* The white blood cell count was 11,800/mm with 78% mature polymorphs, 5% band forms, 17% lymphocytes and 4 / monoytes. Chest X ray showed right middle and lower lobe infiltrates with a small right-sided effusion. Gram stain of the pleural fluid showed gram-positive cocci in clusters (subsequent culture confirmed *Staphylococcus a eus*) The patient was started on oxacillin 2 g intravenously every 6 h w th rapid clinical response. Subsequently there was gradual resolution of the pneu monia and effusion on chest X ray The patient became afebrile and, symptomatically was improved.

*Subsequent laboratory findings included AA phenotype on hemoglobin electro*phoresis, normal serum B12 and fol te levels, serum iron 90 mg dl and total iron binding capacity 315 mg dl. A bone marrow spiration (f g. 1) was performed on the 13th hospital day because of persistent unexplained anemia (hematocrit 4 / ) The marrow was normocellular with a myeloid to erythroid ratio of 2 1 Granulocytic maturation was normal and ron stores were absent. On the ‥nd hospital day the patient developed a fever to 103 F with a shaking ch ll. Physical examination was unremarkable except for sinus tachycardia w th no obvious sites of infection. Chest X ray showed further improvement On the 24th hospital day the patient was still

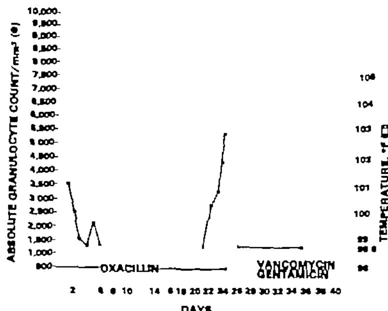



*Fig* 2. Case No. 1 Clinical course showing relationship of absolute granulocyte count, fever and therapy Note rapid defervescence, and rising granulocyte count following discontinuation of oxacillin

febrile and profound granulocytopenia was noted (fig 2) The hematocrit was 26% with normal red cell morphology the white blood cell count was 1,100/mm with 1% mature polymorphs, 26% band forms, 1 promyelocytes and 7% lymphocytes. The platelet count was 300,000/mm and the reticulocyte count 4% A second bone marrow done to evaluate the granulocytopenia showed hypercellularity myeloid to erythroid ratio of 6:1 and striking predominance of early granulocytic precursors (fig 3) Oxacillin was discontinued and the patient was started on vancomycin and gentamicin. The fever promptly subsided, and 24 h later the granulocyte count started to rise (fig 2), with rapid return to normal levels Cultures of the blood, sputum, urine and catheter tips were sterile and vancomycin and gentamicin were discontinued Leukoagglutinins were not detectable (*vide infra*) Thereafter the patient continued to do well and was discharged on the 33rd hospital day She has maintained her pregnancy without recurrence of granulocytopenia

## *Case 2*

A 22-year-old Black male heroin addict was admitted to the Boston City Hospital Medical Service with 103 F temperature, right wrist arthritis and left wrist

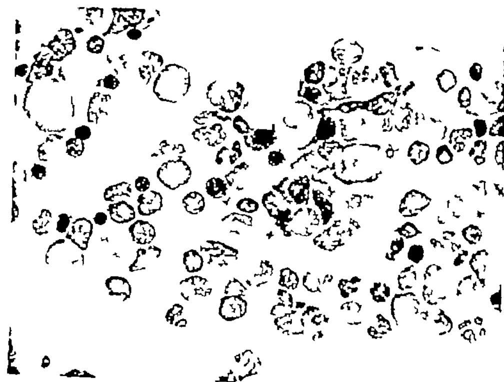


*Fig 1* Initial bone marrow (case No. 1) shows normal myeloid maturation. Wright's Giemsa stain × 600

tory studies showed normal renal function, electrolytes and liver function. The hematocrit was 31 with normal red blood cell indices and adequate platelets. The white blood cell count was 11,800/mm with 78% mature polymorphs, 5% band forms, 12% lymphocytes and 4% monoytes. Chest X ray showed right middle and lower lobe infiltrates with a small right sided effusion. Gram stain of the pleural fluid showed gram-positive cocci in clusters (subsequent culture confirmed *Staphylococcus aureus*). The patient was started on oxacillin 2 g intravenously every 6 h with rapid clinical response. Subsequently there was gradual resolution of the pneumonia and effusion on chest X-ray. The patient became afebrile and symptomatically was improved.

Subsequent laboratory findings included AA phenotype on hemoglobin electrophoresis, normal serum B12 and folate levels, serum iron 90 mg/dl and total iron binding capacity 315 mg/dl. A bone marrow aspiration (fig. 1) was performed on the 13th hospital day because of persistent unexplained anemia (hematocrit 24%). The marrow was normocellular with a myeloid to erythroid ratio of 1. Granulocytic maturation was normal and iron stores were absent. On the 22nd hospital day the patient developed a fever to 103 F with a shaking chill. Physical examination was unremarkable except for sinus tachycardia with no obvious sites of infection. Chest X ray showed further improvement. On the 24th hospital day the patient was still

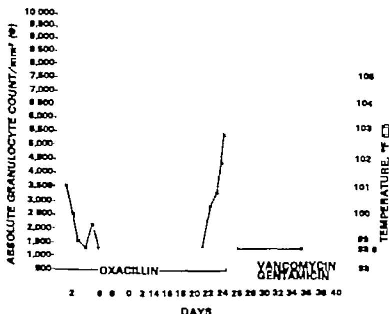



Fig 2. Case No. 1. Clinical course showing relationship of absolute granulocyte count, fever and therapy Note rapid defervescence, and rising granulocyte count following discontinuation of oxacillin.

febrile and profound granulocytopenia was noted (fig. 2). The hematocrit was 26% with normal red cell morphology the white blood cell count was 1 100/mm³ with 1 mature polymorphs, 26% band forms, 1 promyelocytes and 72% lymphocytes. The platelet count was 300,000/mm³ and the reticulocyte count 4%. A second bone marrow done to evaluate the granulocytopenia showed hypercellularity myeloid to erythroid ratio of 6:1 and a striking predominance of early granulocytic precursors (fig 3). Oxacillin was discontinued and the patient was started on vancomycin and gentamicin. The fever promptly subsided, and 24 h later the granulocyte count started to rise (fig 2), with rapid return to normal levels. Cultures of the blood, sputum, urine and catheter tips were sterile and vancomycin and gentamicin were discontinued. Leukoagglutinins were not detectable (*vide infra*). Thereafter the patient continued to do well and was discharged on the 33rd hospital day. She has maintained her pregnancy without recurrence of granulocytopenia.

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A 22-year-old Black male heroin addict was admitted to the Boston City Hospital Medical Service with 103 F temperature, right wrist arthritis and left wrist

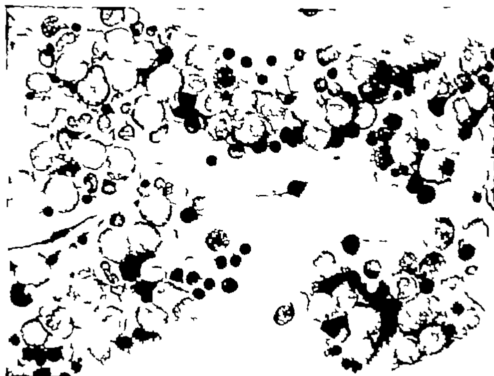


*Fig 3* Repeat bone marrow (case No. 1) during period of profound granulocytopenia shows predominance of immature granulocyte precursors and absence of normal maturation Wright's Giemsa stain. × 600.

tenosynovitis. Admission electrolytes, BUN and liver function tests were within normal limits. The hematocrit was 42%, white blood cell count 7 100/mm³ with 67% polymorphs, 6% lymphocytes and 7% monocytes. With a tentative diagnosis of staphylococcal septicemia, oxacillin 12 g/day intravenously was begun. Cultures of blood and synovial fluid proved positive for *Staphylococcus aureus* sensitive to oxacillin Within 2 days there was a rapid clinical improvement, with defervescence and sterile blood cultures.

On the 21st day the patient's temperature rose to 102 F with associated pharyngitis. No other demonstrable site of infection was noted The throat was not ulcerated. On the 24th hospital day granulocytopenia was noted (fig. 4) The hematocrit was 39% with normochromic, normocytic red blood cells and adequate platelets. The white blood cell count was 2,600/mm³ with 6% polymorphs, 70% lymphocytes, 1% monocytes and 23% eosinophils. The bone marrow aspirate was normocellular with a moderate left shift of the myeloid series (fig. 5). Oxacillin was discontinued and cefalotin, 6 g/day intravenously was started There was rapid defervescence rise in the total granulocyte count within 24 h and disappearance of eosinophilia (fig. 4) Urine, throat and blood cultures were normal. The patient has since re-

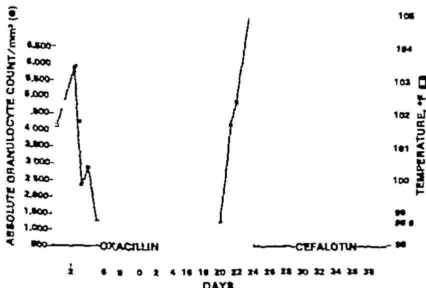



*Fig 4* Case No 2. Clinical course showing relationship of absolute granulocyte count, *fever and therapy. Again note the rapid defervescence and rising granulocyt* count following discontinuation of oxacillin

covered without recurrence of granulocytopenia. Leukoagglutinins were likewise not detectable in the patient serum (*vide infra*).

## Method

Frozen serum was examined for the presence of leukoagglutinins using modification of the method described by HOFFMAN *et al.* [9] The patient' serum was mixed with type-specific normal leukocytes and observed for agglutination. The test was performed both with and without added oxacillin (from the batch that had been administered to the patients). Serum was inactivated at 56 °C for 30 min. Leukocytes were *obtained from type O donors by sedimentation with 6% dextran.* 15 ml of defibrinated blood was mixed with 5 ml of 6% dextran and allowed to stand for 2 h at room temperature. The leukocyte-rich supernatant was removed and centrifuged at 120 g for 5 min The upper portion of the supernatant was removed leaving 2 ml f leukocyte-rich suspension with WBC ranging between 10,000 and 20,000/mm³ The test was performed promptly after harvesting of the leukocytes. Into se ries of 10 × 75 test tubes was placed 0.1 ml of the patient's serum, 0.1 ml of the leukocyte suspension and 0.1 of either saline or oxacillin solution (this was tested at



Fig 5 Bone marrow (case No. ) shows increased myeloid activity with predominance of band form precursors and absence of segmented polymorphs. Wright Giemsa stain. × 600

concentrations of 16.5 and 0.65 mg/ml) The tubes were mixed and incubated 1 h at 37 °C One drop of 1 / acetic acid was added and then one drop of solution was placed on a clean slide and examined under low power for leukoagglutination. Controls showing agglutination were run simultaneously

## *Discussion*

In these 2 patients the occurrence of acute granulocytopenia fever eosinophilia and myeloid maturation arrest are highly suggestive of drug-induced granulocytopenia Both patients experienced a precipitous fall in the granulocyte count and spiking fevers afer 3 weeks of therapy with high dose parenteral oxacillin The nadir of granulocytopenia occurred 24 h after discontinuation of oxacillin with subsequent rapid normalization of the absolute granulocyte count, and remission of fever This striking temporal relationship suggests that granulocyte destruction was due to

*Table I* Granulocytopenia from penicillinase-resistant penicillins

| Drug | Bone marrow | Leukocyte antibodies |
|---|---|---|
| Methicillin [1] | hyperplastic left shift | 0 |
| Methicillin[a] [2] | hyperplastic left shift | 0 |
| Methicillin [3] | non-diagnostic | 0 |
| Methicillin [4] | hyperplastic left shift | |
| Nafcillin [5] | hyperplastic left shift | |
| Nafcillin [6] | hyperplastic left shift | 0 |

0 = Not done – = negative + = positive
[a] 2 cases.

anti-leukocyte antibodies, specifically caused by oxacillin. Recurrent sepsis was excluded as a possible explanation for granulocytopenia by the absence of clinical findings and repeated negative bacteriologic cultures. Transient eosinophilia observed in one patient, further indicates an allergic rather than infectious process and has been reported previously in drug-induced granulocytopenia [10].

There are two well-defined mechanisms of drug-induced granulocytopenia. The first and most common is direct bone marrow suppression as occurs with chlorpromazine [11], sulfonamides [12] and thiouracil [13]. In this situation the bone marrow is often hypocellular, generally with a more pronounced depression of the myeloid series. Less frequently, administration of a drug results in formation of anti-granulocyte destruction. This was first described in aminopyrine-induced granulocytopenia [14]. The bone marrow is typically hyperplastic with an abundance of early granulocytic precursors, so-called 'maturation arrest'. Although one might expect to demonstrate leukoagglutinins easily under those circumstances, they are usually not detectable, as in our 2 patients.

*Leukoagglutinins have been demonstrated in penicillin-induced granulocytopenia* [8]. However, in the 8 reported cases of granulocytopenia secondary to semisynthetic penicillins, leukoagglutinins were not present (table I). In most of these cases of semisynthetic penicillin-related granulocytopenia the bone marrow has been reported hyperplastic rather than hypocellular suggesting an immunologic mechanism of peripheral granulocyte destruction. This would appear to be the mechanism in our 2 patients treated with oxacillin.



Fig 5 Bone marrow (case No. 2) shows increased myeloid activity with predominance of band form precursors and absence of segmented polymorphs. Wright Giemsa stain × 600

concentrations of 16.5 and 0.65 mg/ml) The tubes were mixed and incubated 1 h at 37 C One drop of 1 / acetic acid was added and then one drop of solution was placed on a clean slide and examined under low power for leukoagglutination. Controls showing agglutination were run simultaneously

## Discussion

In these 2 patients the occurrence of acute granulocytopenia fever eosinophilia and myeloid maturation arrest are highly suggestive of drug-induced granulocytopenia Both patients experienced a precipitous fall in the granulocyte count and spiking fevers afer 3 weeks of therapy with high dose parenteral oxacillin The nadir of granulocytopenia occurred 24 h after discontinuation of oxacillin with subsequent rapid normalization of the absolute granulocyte count, and remission of fever This striking temporal relationship suggests that granulocyte destruction was due to

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# Coagulation and Fibrinolysis in Thyroid Disease

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*Key Words.* Antitrypsin · $\alpha_2$-Macroglobulin · Antithrombin III · Blood coagulation · C1 inactivator · Fibrinogen · Fibrinolysis · Plasminogen · Thyroid disease

*Abstract.* The levels of components of the coagulation mechanism and fibrinolytic system in 20 hyperthyroid patients and 9 hypothyroid patients were compared with those of 20 euthyroid control subjects. The mean levels of fibrinolytic activity and plasminogen were significantly reduced in the hyperthyroid patients while mean levels of $\alpha_1$-antitrypsin and C1 inactivator were increased. Patients with hypothyroidism had significantly increased levels of fibrinolytic activity and $\alpha_2$-macroglobulin, prolonged partial thromboplastin time, and reduced levels of factor XII and antithrombin III.

A number of hormonal influences on components of the haemostatic mechanism are known; the effect of oestrogens on blood coagulation and the increased blood fibrinolytic activity induced by adrenaline have received particular study in recent years. The effect of thyroid hormones on haemostasis has attracted relatively little attention although alterations in components of both the fibrinolytic enzyme system and the coagulation mechanism have been reported in patients with thyroid dysfunction. Hyperthyroidism has been shown to be associated with decreased fibrinolytic activity [4, 16], decreased plasminogen concentration [4], increased factor VIII levels [11, 14, 31] and a decreased level of the vitamin K-dependent factors [3, 17]. In contrast, hypothyroidism is associated with increased fibrinolytic activity [16, 18] and a number of patients have a decrease in the levels of factors VIII, IX and XI [12, 31]. In order to elucidate the mechanism of some of these changes and extend our knowledge of the effect of thyroid hormones on the haemostatic mechanism we have

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## Results

### Fibrinolysis

Fibrinolytic activity was significantly decreased in the hyperthyroid patients. A negative correlation ($r = -0.4628$ $p < 0.05$) existed between fibrinolytic activity and the percentage [131]I thyroid uptake in 4 h, but the relationship between fibrinolytic activity and the PBI was not significant. In contrast to the hyperthyroid patients, those with hypothyroidism had a mean level of fibrinolytic activity significantly greater than the control subjects. In the case of hypothyroid patients fibrinolytic activity did not correlate with either the PBI or the percentage radioactive iodine gland uptake.

Fibrinolytic capacity did not differ significantly between the 3 groups, but plasminogen levels were significantly lower in the patients with hyper thyroidism. Fibrinogen and FR-antigen levels did not differ significantly between the hyperthyroid or hypothyroid patients and the control subjects.

### Protease Inhibitors

The mean levels of both -antitrypsin and C1 inactivator were increased in patients with hyperthyroidism. Patients with hypothyroidism had a higher mean level of $\alpha_2$-macroglobulin and a reduced level of antithrombin III.

### Coagulation System

The hypothyroid patients had a significantly prolonged mean partial thromboplastin time. Factor X and XI did not differ significantly between the groups, but the mean factor XII level was significantly lower in the hypothyroid patients. In these patients the factor XII level was not significantly correlated with fibrinolytic activity serum aspartate aminotransferase, serum bilirubin, serum alkaline phosphatase or serum albumin levels.

### Liver Function Tests

No patient studied, either hypothyroid or hyperthyroid, had a gross abnormality in liver function. 11 of the 29 patients had an aspartate aminotransferase level between 15 and 22 U/l, 12 patients had an alkaline phosphatase level between 100 and 200 IU/l, while no patient had a serum bilirubin level in excess of 16 $\mu$mol/l. The lowest serum albumin level was 31 g/l. However the mean aspartate aminotransferase level in both hyperthyroid patients of 14.3 U/l (SD $\pm$ 4.4) and hypothyroid patients (14.3 $\pm$ 2.8 U/l) was significantly greater ($p < 0.05$) than that of

studied a number of components of the fibrinolytic and coagulation systems in hyperthyroid and hypothyroid patients and in euthyroid control subjects

## Materials and Methods

*Fibrinolytic activity* was measured in the plasma separated from venous blood obtained with minimal venous stasis using the euglobulin clot lysis time method [23] and a clot lysis time recorder (Carmanan Instrumentation Ltd, Glasgow). The results were expressed by plotting the lysis times logarithmically against units of fibrinolytic activity [30] 10 units being arbitrarily equated with a lysis time of 50 min. This technique is primarily a measure of plasminogen activator activity

*Fibrinolytic capacity* the release of plasminogen activator in response to venous occlusion, was assessed as described previously [?9]

*Plasminogen* was measured by the caseinolytic technique of ALKJAERSIG et al [2], and the results expressed in Sherry units [1]

*Fibrinogen* was measured by a modification [24] of the method of RATNOFF and MENZIE [28]

*Fibrinogen-related antigen (FR-antigen)* was assayed by the tanned red cell haemagglutination-inhibition technique of MERSKEY et al [?0]

*α-Macroglobulin α-antitrypsin antithrombin III and C1 inactivator* levels were measured by single radial immunodiffusion [19] using reagents obtained from Hoechst Pharmaceuticals. The level of C1 inactivator was expressed as a percentage of the level in a plasma pool prepared from 20 healthy donors.

*Factors X XI and XII* were assayed by one-stage techniques based on the PROCTER and RAPAPORT [26] using platelet substitute obtained from General Diagnostic plasmas [10, 28] A normal plasma pool obtained from 20 healthy male medical students was used as a control

*Partial thromboplastin time* was measured by a modification of the method of PROCTOR and RAPAPORT [ 6] using platelet substitute obtained from General Diagnostic Dept., Warner & Co Ltd., Eastleigh.

*Serum protein-bound iodine (PBI) and serum bilirubin alkaline phosphatase aspartate aminotransferase and protein* assays were performed by standard techniques in the Department of Chemical Pathology University of Aberdeen. *Thyroidal radioactive iodine uptake studies* were undertaken by the Department of Medical Physics, University of Aberdeen

*Patients* the 49 patients studied had been referred to the thyroid clinic on account of a suspected disorder of thyroid function. The patients were categorised according to the findings on clinical examination, and the results of the PBI and 4 h $^{131}$I thyroid uptake. Patients with other major pathology and those receiving steroids, adrenoceptor agonists or antagonists, or other drugs known to alter the measured parameters were not included.

The ?0 patients with hyperthyroidism (18 women and 2 men) had an age range of 34–68 (mean 54 ± 11) years. The 9 hypothyroid patients (6 women and 3 men) had an age range of 41 74 (mean 59 ± 9) years, while the euthyroid controls were 19 women and 1 man with an age range of 24–78 (mean 48 ± 16) years.

ism and hypothyroidism [32] while changes in components of the fibrinolytic system have been observed in liver disease [13 ?1 ·5] However the absence of a relationship between fibrinolytic activity and plasminogen and indices of liver function suggests that the changes found in patients with thyroid dysfunction are not mediated by liver disease.

The relationship between indices of thyroid overactivity and fibrinolytic activity was not close and it seems unlikely that changes in fibrinolysis are a direct consequence of an altered blood thyroid hormone level. The recent observation contrary to previous views, that patients with hyperthyroidism possibly have decreased sympathetic activity [6] may be relevant to the fibrinolytic activity reduction since there is abundant evidence for the induction of fibrinolytic activity by catecholamines [5 30]

Plasma contains a number of protease inhibitors shown by *in vitro* studies to be capable of inhibiting plasmin These include $\alpha_2$ macroglobulin, $\alpha$ -antitrypsin, antithrombin III and C1 inactivator Previous investigators [4] have shown that total antiplasmin activity is increased in patients with hyperthyroidism Examination of the level of the individual protease inhibitors in this study shows that both $\alpha$ -antitrypsin and C1 inactivator were increased in hyperthyroid patients. In contrast, patients with hypothyroidism had a higher mean level of $\alpha_2$-macroglobulin.

Recent assessment of the relative importance of protease inhibitors in plasma antiplasmin activity has suggested that $\alpha_2$-macroglobulins is the major antiplasmin [15] but $\alpha$ -antitrypsin also has a role in the progressive antiplasmin activity [8] A further -globulin immediate plasmin inhibitor *not measured in this study* has been identified [7] and this inhibitor rather than $\alpha$ -antitrypsin may be responsible for the increased antiplasmin activity in patients with hyperthyroidism. The explanation for the lack of a reported increase in antiplasmin activity in hypothyroidism in the light of the present finding of raised levels of $\alpha_2$-macroglobulin is uncertain.

SIMONE *et al* [31] observed a prolongation of the partial thromboplastin time in patients with hypothyroidism and specific factor assays revealed a number of patients with reduced levels of factors VIII IX and XI The present study has concentrated on the contact factors XI and XII We confirmed the prolonged partial thromboplastin times in hypothyroid patients, together with a significant reduction in the mean factor XII level, but without change in the mean factor XI concentration. The decrease in coagulation factors may merely be the result of the general decrease in protein synthesis found in hypothyroidism [9]

*Table I* Mean levels of components of the fibrinolytic and coagulation system in control subjects and in hyperthyroid and hypothyroid patients (± SD)

| | Controls n = 20 | Hyperthyroid n = 20 | Hypothyroid n = 9 |
|---|---|---|---|
| Fibrinolytic activity U | 3 8 ± 1 8 | [illegible]6 ± 1 6 | 5 7 ± 2.8 |
| Fibrinolytic capacity U | 15 0 ± 6.8 | 14.2 ± 9 1 | 17.2 ± 10.7 |
| Plasminogen, U/ml | 4 1 ± 0.5 | 3.5 ± 0.5 | 4.[illegible] ± 0.7 |
| FR-antigen, µg/ml | 6.2 ± 7.5 | 5.2 ± 4 4 | 6.5 ± 6.1 |
| Fibrinogen mg/dl | 4[illegible]2 ± 94 | 432 ± 83 | 486 ± 96 |
| $\alpha_1$ Antitrypsin, mg/dl | 295 ± 65 | 390 ± 102 | 30[illegible] ± 93 |
| $\alpha_2$-Macroglobulin mg/dl | 349 ± 99 | 346 ± 109 | 517 ± 154 |
| Cl inactivator ⁄ | 118 ± 31 | 160 ± 53 | 138 ± 40 |
| Antithrombin III mg/dl | 46.0 ± 5 9 | 44.[illegible] ± 13.0 | 37.2 ± 5.1*** |
| PTT (ratio) | 0.90 ± 0 16 | 0.89 ± 0.16 | 1 16 ± 0.23*** |
| Factor X, | 120 ± 54 | 115 ± 42 | 105 ± 38 |
| Factor XI % | 103 ± 30 | 115 ± 35 | 114 ± 31 |
| Factor XII % | 108 ± 40 | 104 ± 42 | 71 ± 32** |

Comparison to controls $p < 0.05$ $p < 0.01$ $p < 0.005$ $p < 0.001$

the control subjects (11 6 ± 2.8 U/l) and the mean level of alkaline phosphatase in the hyperthyroid patients (117 ± 43 7 IU/l) was also higher than that of the control subjects (65.5 ± 24 4) this difference being highly significant ($p < 0\ 001$) Serum albumin levels were significantly lower ($p < 0\ 005$) in the hyperthyroid patients (35 8 ± 2.9 g/l) than in the euthyroid controls (39 4 ± 4 0 g/l) When hypothyroid and hyperthyroid groups were analysed separately no significant correlation was found between either fibrinolytic activity or plasminogen level and the levels of aspartate aminotransferase alkaline phosphatase, bilirubin or albumin

## *Discussion*

This study has confirmed previous observations that plasma plasminogen activator levels are reduced in patients with hyperthyroidism and increased in those with hypothyroidism [4 16] although it may be noted that NAPIORKOWSKA *et al* [22] found very high fibrinolytic activity in patients with severe thyrotoxicosis. The normal fibrinolytic capacity found in the present investigation suggests that these alterations in fibrinolytic activity do not result from changes in the synthesis or release of activator

Hepatic dysfunction is a recognised occurrence in both hyperthyroid

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Although precise statistics are not available it has been a clinical impression that thromboembolic disorders are uncommon in patients with hyperthyroidism [3 14 17] This is of interest in view of the reduction in plasma fibrinolytic activity and increase in two of the plasma protease inhibitors with antiplasmin activity but the hyperdynamic circulation [3] may counterbalance these potential hypercoagulable changes. While arterial disease is prevalent in untreated hypothyroidism there is no known evidence for an increased incidence of clinical thromboembolism. It is possible that the relatively increased fibrinolytic activity in such patients may have a protective role

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## *Material and Methods*

20 patients with β-thalassemia intermedia in 18 families were studied. The ages of the patients varied between 7 and 47 years with mean of 21.7. The hematological methods were all standard. Blood counts were performed mostly by coulter counter model ZF. Hemoglobin analyses were carried out by starch-gel electrophoresis using Tris-edta-borate buffer at pH 8.6 [25]. Quantitative estimations of hemoglobin $A_2$(Hb-$A_2$) were performed by the methods of DEAE- or DE-cellulose chormatographies [11, 15]. The upper limit for Hb-$A_2$ in these methods are 3 and 3.2%, respectively. Fetal hemoglobin (Hb-F) was estimated by using alkali denaturation technic [22]. Glucose-6-phosphate dehydrogenase activity was determined qualitatively by a screening method using Dade reagents (Miami, Fla.). Intraerythrocytic and Heinz bodies were examined by the incubation of red cells at 37 °C with cresyl blue [10]. Haptoglobin was determined by the method of polyacrylamide-gel electrophoresis [17]. Distribution of Hb-F in red blood cells was determined by the method of acid elution [16].

## *Results*

*β-Thalassemia intermedia homozygous for β-thalassemia with increased Hb-$A_2$.* Table I gives some clinical, hematological and genetic data of 9 patients. All the patients had moderate clinical and hematological findings of a thalassemia syndrome. The absence or scarcity of transfusion requirement was the most noteworthy clinical finding. 3 patients (cases 1 3 8) have undergone splenectomy due to hypersplenism during their hospital stay. Hemoglobin F varied between 11 and 70% with a mean of 40.7%. In 8 patients, genetic studies showed that both parents were β-thalassemia heterozygotes with increased Hb-$A_2$. In the last patient (case 9) only one of the parents was studied. Despite this, we accepted this patient as possibly homozygous for β-thalassemia with increased Hb-$A_2$, because all her 3 children were β-thalassemia heterozygotes with increased Hb-$A_2$, whilst her husband was normal.

*β-Thalassemia intermedia homozygous for β-thalassemia with normal levels of Hbs. A and F.* Table II gives some clinical and hematological data of the family members of 3 patients with this syndrome. They disclosed moderate clinical, hematological and mild roentgenographic findings of a thalassemia syndrome. 1 of the patients (case 12) had his first transfusion at an earlier age than the others, at 10, possibly due to superimposed infections. Both parents of the patients showed all hematologic findings of β-thalassemia with normal levels of Hbs $A_2$ and F.

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# Different Types of Beta-Thalassemia Intermedia

## A Genetic Study in 20 Patients[1]

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*Key Words* β-Thalassemia intermedia Types of β-thalassemia 'Silent β-thalassemia genes

*Abstract* 20 patients with β thalassemia intermedia classified according to the results of genetic studies are presented. (1) 9 patients with β-thalassemia intermedia homozygous for β thalassemia with increased $Hb\text{-}A_2$ are reported. (2) 8 patients with β-thalassemia intermedia, 3 homozygous for β-thalassemia with normal levels of Hbs A and F 5 heterozygous for both this and β-thalassemia with increased $Hb\text{-}A_2$ are presented. (3) 2 families with β-thalassemia intermedia heterozygous for both β thalassemia with increased $Hb\text{-}A_2$ and silent β-thalassemia are reported. Two different varieties are presented.

Although the term of thalassemia intermedia is ill defined, it is generally used. As we stated previously [3] the criteria for the diagnosis of this thalassemia syndrome are as follows (1) the clinical manifestations and hematological findings are moderate in degree (2) the absence or scarcity of transfusion requirements, and (3) mostly the ages of thalassemia patients are over 20

Recently we have studied 20 cases of β-thalassemia intermedia. They were in conformity with all the criteria forwarded above. The results of the genetic study in each case of β-thalassemia intermedia varied widely In this paper we report the genetic variations in these patients.

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## Material and Methods

20 patients with β-thalassemia intermedia in 18 families were studied. The ages of the patients varied between 7 and 47 years with mean of 21.7 The hematological methods were all standard. Blood counts were performed mostly by a coulter counter, model ZF Hemoglobin analyses were carried out by starch-gel electrophoresis using Tris-edta-borate buffer at pH 8.6 [25]. Quantitative estimations of hemoglobin $A_2$(Hb-$A_2$) were performed by the methods of DEAE- or DE-cellulose chromatographies [11, 15]. The upper limit for Hb-$A_2$ in these methods are 3 and 3.2%, respectively Fetal hemoglobin (Hb-F) was estimated by using alkali denaturation technic [22]. Glucose-6-phosphate dehydrogenase activity was determined qualitatively by screening method using Dade reagents (Miami, Fla.). Intraerythrocytic and Heinz bodies were examined by the incubation of red cells at 37 °C with cresyl blue [10]. Haptoglobin was determined by the method of polyacrylamide-gel electrophoresis [17]. *Distribution of Hb-F in red blood cells was determined by the method* of acid elution [16].

## Results

*β-Thalassemia intermedia homozygous for β-thalassemia with increased Hb-$A_2$.* Table I gives some clinical, hematological and genetic data of 9 patients. All the patients had moderate clinical and hematological findings of a thalassemia syndrome. The absence or scarcity of transfusion requirement was the most noteworthy clinical finding. 3 patients (cases 1 3 8) have undergone splenectomy due to hypersplenism during their hospital stay Hemoglobin F varied between 11 and 70% with a mean of 40 7 %. In 8 patients, genetic studies showed that both parents were β-thalassemia heterozygotes with increased Hb-$A_2$. In the last patient (case 9) only one of the parents was studied. Despite this, we accepted this patient as possibly homozygous for β-thalassemia with increased Hb-$A_2$, because all her 3 children were β-thalassemia heterozygotes with increased Hb-$A_2$, whilst her husband was normal.

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*Table I* Some clinical, hematological and genetic data in 9 patients with β-thalassemia intermedia homozygous for β-thalassemia with increased Hb-$A_2$

| Case No. | Age, years | sex | Hepato-spleno-megaly | Trans-fusion require-ments | Radio-logic bone changes | RBC × $10^6/\mu l$ | Hb g% | Hct % | MCV $\mu m^3$ | MCH pg | Morph-ologic RBC changes | NRBC/100 WBC | Serum Iron µg% | Hb-F % | Hb-$A_2$ % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 21 | F | ++++ | never | ++ | 1.10[1] | 2.3 | 8 | 73 | 20 | ++++ | 40 | 181 | 40.0 | 2.9 |
| Mo | 40 | | – | never | – | 5.20 | 12.2 | 42 | 80 | 24 | + | 0 | 100 | 0.0 | 4.2 |
| Fa | 40 | | – | never | – | 5.00 | 10.7 | 39 | 78 | 21 | + | 0 | 90 | 0.0 | 4.1 |
| 2 | 14 | M | ++ | never | + | 3.10 | 7.0 | 25 | 80 | 22 | +++ | 2 | 175 | 49.0 | 1.7 |
| 3 | 16 | M | +++ | never | ++ | 2.00[1] | 5.0 | 15 | 75 | 25 | +++ | 0 | 100 | 31.0 | 3.2 |
| Mo | 35 | | – | never | – | –[3] | –[3] | | –[3] | –[3] | ++ | 0 | –[3] | 0.0 | 4.1 |
| Fa | 40 | | – | never | – | 5.60 | 13.0 | 45 | 80 | 25 | ++ | 0 | 90 | 0.0 | 4.7 |
| 4 | 16 | F | – | rarely | +++ | 3.30 | 6.7 | 25 | 75 | 20 | +++ | 220 | 140 | 30.5 | 4.8 |
| Mo | 41 | | – | never | – | 5.00 | 11.2 | 38 | 76 | 22.4 | ++ | 0 | 95 | 0.0 | 4.6 |
| Fa | 48 | | – | never | – | 5.56 | 11.8 | 47 | 83 | 21 | ++ | 0 | 90 | 0.0 | 4.7 |
| 5 | 7 | M | + | never | + | 3.00 | 7.0 | 22 | 73 | 23 | +++ | 0 | 100 | 44.0 | 2.1 |
| Mo | 26 | | – | never | – | 4.40 | 11.4 | 35 | 79.5 | 26 | ++ | 0 | 80 | 0.0 | 4.2 |
| Fa | 30 | | – | never | – | 5.00 | 12.0 | 41 | 82 | 24 | ++ | 0 | 96 | 0.0 | 4.8 |
| 6 | 22 | M | –[3] | rarely | ++++ | 3.30 | 6.0 | 24 | 73 | 18 | ++++ | 610 | 120 | 25.0 | 2.3 |
| Mo | 47 | | – | never | – | 5.30 | 12.8 | 40 | 75 | 24 | ++ | 0 | 92 | 0.0 | 4.0 |
| Fa | 52 | | – | never | – | 5.60 | 13.0 | 44 | 78.5 | 23 | + | 0 | 102 | 0.0 | 4.4 |

*Table I* (continued)

| Case No. | Age, years | sex | Hepato-splenomegaly | Transfusion requirements | Radiologic bone changes | RBC $10^6/\mu l$ | Hb g% | Hct % | MCV µm | MCH pg | Morphologic RBC changes | NRBC/100 WBC | Serum Iron µg% | Hb-F % | Hb-$A_2$ % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7 | 15 | F | – | rarely | + + + + | 2.90 | 6.2 | 22 | 76 | 21 | + + + + | 65 | 110 | 70.0 | 1.8 |
| Mo | 48 | | – | never | – | 5.20 | 12.1 | 40 | 77 | 23 | + + | 0 | 80 | 0.0 | 4.0 |
| Fa | 52 | | – | never | – | 5.80 | 13.2 | 46 | 79 | 23 | + | 0 | 93 | 0.0 | 4.4 |
| 8 | 18 | M | + + | never | – | 3.80 | 8.7 | 31 | 81 | 23 | + + + | 1 | 93 | 67.0 | 1.7 |
| Mo | 32 | | – | never | – | 5.00 | 9.4 | 39 | 78 | 19 | + + | 0 | 80 | 4.5 | 4.7 |
| Fa | 38 | | – | never | – | 5.30 | 11.2 | 43 | 81 | 21 | + + | 0 | 86 | 0.0 | 5.7 |
| 9 | 47 | F | – | rarely | + + + | 3.50 | 6.3 | 27 | 77 | 18 | + + + | 6 | 200 | 11.0 | 5.7 |
| Fa | 68 | | – | never | – | 5.10 | 12.3 | 42 | 82 | 24 | + | 0 | 120 | 0.0 | 4.2 |
| D | 24 | | – | never | – | 4.30 | 12.1 | 34 | 79 | 26 | + + | 0 | 89 | 0.0 | 4.0 |
| S | 22 | | – | never | – | 4.90 | 12.2 | 40 | 81 | 25 | + + | 0 | 94 | 0.0 | 4.7 |
| D | 18 | | – | never | – | 5.20 | 13.0 | 41 | 79 | 25 | + + | 0 | 82 | 0.0 | 4.4 |
| H | 51 | | – | never | – | 5.20 | 14.1 | 47 | 90 | 27 | – | 0 | 98 | 0.0 | 2.8 |

Mo = Mother Fa = Father D = daughter S = son H = husband. Cases 1 and 2 are brother and sister
Hematologic data before splenectomy
Not performed.
Splenectomized previously

*Table II* Some clinical, hematological and genetic data in 3 patients with β-thalassemia intermedia homozygous for β-thalassemia with normal levels of hemoglobins $A_2$ and F

| | Case 10[1] | Mo | Fa | Case 11 | Mo | Fa | Case 12 | Mo | Fa |
|---|---|---|---|---|---|---|---|---|---|
| Age (years), sex | 24 F | 43 | 46 | 19 M | 40 | 46 | 10 M | 37 | 41 |
| Hepatosplenomegaly | + + + | – | – | + + | – | – | + + | – | – |
| First transfusion at the age of | 24 | – | – | – | – | – | 10 | – | – |
| Transfusion requirement | very rarely | never | never | never | never | never | rarely | never | never |
| Radiologic bone changes | + | – | – | + + | – | – | + | – | – |
| RBC × $10^6$/µl | 3.20 | 5.21 | 5 45 | 4 00 | 4 80 | 5 00 | 3.20 | 4 40 | 4.70 |
| Hb, g% | 6.0 | 12.8 | 12.9 | 7 8 | 11 8 | 1 1 | 7 0 | 11 1 | 1.5 |
| Hematocrit, % | 20 | 42 | 4[illegible] | 32 | 38 | 38 | 26 | 36 | 36 |
| MCV $\mu^3$ | 62.5 | 80.7 | 76 | 80 | 79 | 76 | 81 | 82 | 76.5 |
| MCH, pg | 19 | 24 6 | 23 4 | 19.5 | 24 | 24 | 22 | 25 | 26.5 |
| Morphologic RBC changes | + + + | + + | + + | + + + | + | + | + + + | + + | + |
| NRBC/100 WBC | 0 | 0 | 0 | 1 | 0 | 0 | 8 | 0 | 0 |
| Serum iron, µg% | 93 | 88 | 98 | 190 | 84 | 92 | 84 | 127 | 143 |
| Hb-F % | 15 | 0 | 0 | 10.5 | 0 | 0 | 10 | 0 | 0 |
| Hb-$A_2$, % | 5 | 3.1 | 2.3 | 5.8 | 2.1 | 2.3 | 6 | 2 | 2.3 |

[1] The patient had the findings of hypersplenism such as mild leukopenia and thrombocytopenia. Pancytopenia disappeared following splenectomy

*β-Thalassemia intermedia heterozygous for both β-thalassemia with increased Hb $A_2$ and β-thalassemia with normal levels of Hbs $A_2$ and F*

Some clinical, hematological and genetic data of 5 patients with this syndrome are given in table III The absence or scarcity of transfusion requirement was noted in all the patients. 1 of them (case 14) had her first transfusion at the age of 40 when the signs of hemachromatosis appeared. The other 1 (case 13) had her first transfusions only during her 2 pregnancies. In 3 patients (cases 14 16 17) due to hypersplenism splenectomy was performed In 2, hematologic status improved following splenectomy But in 1 patient (case 14) due to presence of hemachromatosis, no satisfactory change in hematologic data was observed. Family studies showed that one parent had the findings of β-thalassemia with increased

*Table III* Some clinical, hematological and genetic data in 5 patients with β-thalassemia intermedia heterozygous for both β-thalassemia with increased Hb-$A_2$ and β-thalassemia with normal levels of Hbs, $A_2$ and F

| Case No. | Age, years sex | Hepato-splenomegaly | First transfusion at the age of | Transfusion requirements | Radiologic bone changes | RBC × $10^6/\mu l$ | Hb g % | Hct % | MCV $\mu^3$ | MCH pg | Morphologic RBC changes | NRBC/ 100 WBC | Serum iron µg % | Hb-F % | Hb-$A_2$ % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | 32 F | + | 25 | rarely | + | 3.60 | 6.6 | 28 | 78 | 18 | + + + | 0 | 150 | 11 | 5.8 |
| M | 54 | – | | never | – | 5 13 | 11.5 | 43 | 83 | 22 | + | 0 | 100 | 0.5 | 3.0 |
| F | 62 | | | never | – | 4.90 | 9 4 | 40 | 81.6 | 19 | + | 0 | 95 | 0 0 | 4.8 |
| 14 | 40 F | + + + | 40 | very rarely | + | 3.20 | 7.0 | 26 | 81 | 23 | + + + | 0 | 108 | 30 0 | 1 9 |
| Mo | 65 | | | never | – | 5.20 | 11.3 | 41 | 79 | 22 | + | 0 | | 0 0 | 4.2 |
| F | 85 | – | | never | | 5 00 | 12 | 40 | 80 | 24 | + | 0 | 93 | 0 0 | 2.1 |
| 15 | 26 F | + + + + | 18 | rarely | + + | 3 20 | 5.2 | [illegible] | 72 | 16 | + + + + | 0 | 125 | 23 0 | 3.0 |
| M | 62 | – | | never | – | 5.10 | 11 7 | 40 | 78 | 23 | + + | 0 | 100 | 1.0 | 2.0 |
| Fa | 57 | | | never | – | 5.40 | 10 9 | 43 | 79 6 | 20 | + + | 0 | 98 | 1.0 | 4.3 |
| 16 | 11 M | | 6 | rarely | + | 3 30 | 6 0 | 26 | 79 | 18 | + + + + | 160 | 140 | 25.0 | 4 0 |
| Mo | 37 | – | | never | – | 4 50 | 9 9 | 36 | 80 | 22 | + | 0 | 100 | 0 0 | 3.0 |
| F | 40 | – | | never | – | 5.10 | 13.0 | 42 | 82 | 26 | + | 0 | 95 | 0.0 | 3 9 |
| 17 | 18 F | – | 5 | ery rarely | + + | 3.10 | 7.0 | 25 | 80 | 22 | + + + | 90 | 135 | 16.4 | 4.8 |
| Mo | 39 | | | never | – | 4.70 | 11.5 | 38 | 81 | 25 | + | 0 | 102 | 0 0 | 3.0 |
| F | 46 | – | | never | – | 4.80 | 11 0 | 37 | 77 | 23 | + | 0 | 90 | 0 0 | 4 0 |

Not performed.
Splenectomized previously

*Table IV.* Some clinical, hematological and genetic data in 3 patients with β-thalassemia intermedia heterozygous for both β-thalassemia with increased Hb-A₂ and 'silent' β-thalassemia

| | Case 18[1] | Case 19 | Mother | Father | Case 20 | Mother | Father |
|---|---|---|---|---|---|---|---|
| Age (years), sex | 23 F | 21 M | 36 | 44 | 34 | 47 | 55 |
| Hepatosplenomegaly | ++++ | ++ | – | – | +++ | – | – |
| First transfusion at the age of | 22 | – | – | – | 19 | – | – |
| Transfusion requirement | very rarely | never | never | never | very rarely | never | never |
| Radiologic bone changes | ++ | + | – | – | + | – | – |
| RBC, $10^6/\mu l$ | [illegible].10 | 4.00 | 4.20 | 4.70 | 3.70 | 4.00 | 4.60 |
| Hb, g% | 3.8 | 7.8 | 12.1 | 10.0 | 7.1 | 9.3 | 13.0 |
| Hematocrit, % | 13 | 31 | 40 | 37 | 26 | 31 | 44 |
| MCV, $\mu^3$ | 62 | 77.5 | 95 | 79 | 70 | 77.5 | 95.6 |
| MCH, pg | 18 | 19.5 | 29 | 21 | 19 | 23 | 26 |
| Morphologic RBC changes | ++++ | +++ | – | ++ | +++ | ++ | – |
| NRBC/100 WBC | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Serum iron, μg% | 180 | 160 | 80 | 128 | 150 | 95 | 100 |
| Hb-F, % | 56 | 62 | 0.2 | 5 | 3.6 | 0 | 0 |
| Hb-A₂, % | 2 | 2.5 | 3.1[2] | 6[2] | 5.3 | 4.1 | 2.6 |

[1] The patient had the findings of a severe hypersplenism such as leukopenia, thrombocytopenia and a severe anemia. Therefore, she was splenectomized and pancytopenia disappeared following splenectomy.

[2] Performed by the method of starch-gel electrophoresis [4].

Hb-A₂ and the other one had those of β thalassemia with normal levels of Hbs A₂ and F.

*β-Thalassemia intermedia heterozygous for both β-thalassemia with increased Hb-A₂ and 'silent' β-thalassemia.* Table IV gives some clinical and hematological data of the family members of each patient. According to the different results of hemoglobin analysis obtained in these 3 patients, 2 varieties of this syndrome are considered.

*Type 1. β-thalassemia intermedia with high level of Hb-F heterozygous for both β-thalassemia with increased Hb-A₂ and 'silent' β-thalassemia.* 2 patients, brother and sister described previously [6] with this syndrome were studied. Both were never transfused till the development of a

severe pancytopenia due to hypersplenism in 1 of them (case 18). Following splenectomy pancytopenia disappeared completely. Family study showed that the father was a β-thalassemic heterozygote with increased Hb-$A_2$ and in the mother however the results of hematologic studies were within the normal range.

*Type 2 β-thalassemia intermedia with slightly elevated Hb-F heterozygous for both β-thalassemia with increased Hb-A and silent β-thalassemia.* Only 1 patient (case 20) with this syndrome was studied. Her family study showed that the mother had the hematologic findings of β-thalassemia with increased Hb-$A_2$ and those of the father were within normal range (parentage was supported by data from extensive blood group typing).

## Discussion

The heterogeneity of the β-thalassemia genes are well known. Despite some attempts to classify these genes [12, 13 25] there is no definite and generally accepted one. Nowadays, it seems that there are only two ways in differentiating β-thalassemia genes from each other: (1) a complete family study and (2) measurement of the relative rate of α/β-chain synthesis using a radioactive amino acid incorporation technique [25]. Unavailability of the latter method forced us to rely on a complete family study in classifying these 20 patients with β-thalassemia intermedia.

There are several papers dealing with homozygous β-thalassemia of the mild variety [3 8, 14 25]. The only interesting point in our patients with this syndrome was the age of 1 of them (case 9). She was still active in her profession at the age of 47

BERNINI *et al.* [9] FESSAS [12] AKSOY and ERDEM [5], and WEATHERALL and CLEGG [25] have already noted the presence of some β-thalassemia genes compatible with normal levels of Hb-$A_2$ and F [7 13]. BERNINI *et al.* [9] published a case of thalassemia intermedia, one of whose parents showed normal levels of Hb-$A_2$ and F. The authors postulated that the propositus was carrying two different allelomorphic variants of β-thalassemia, one of them being associated with normal levels of Hb-$A_2$ and F. Similarly FESSAS emphasized the presence of β-thalassemia genes causing no alterations in the level of Hb-$A_2$ and F among subdivisions of β-thalassemia genes [12, 13]. On the other hand, it is difficult to distinguish a β-thalassemia heterozygote with normal levels of Hb-$A_2$ and F from an α-thalassemia heterozygote. The family study may show the pres

ence of this type of $\beta$-thalassemia gene in the parent with normal levels of Hbs-$A_2$ and F As PEARSON [18] showed clearly that in case of the combination of $\alpha$- and $\beta$-thalassemia, the clinical and hematological findings are no more severe than in either trait alone, this form of thalassemia syndrome can easily be eliminated. Furthermore, in some heterozygotes with $\delta\beta$-thalassemia the levels of Hbs-$A_2$ and F could be found in normal range [12, 13] The only possibility to distinguish this form of $\delta\beta$-thalassemia from $\beta$-thalassemia with normal levels of Hbs-$A_2$ and F is family study As it is known, in the homozygous state for $\delta\beta$-thalassemia or in the heterozygous state for both this and $\beta$ thalassemia, the levels of Hb-F are very high [23 25] Therefore in cases of $\beta$-thalassemia intermedia associated with low levels of Hb-F these syndromes of $\delta\beta$-thalassemia should not be considered Another possibility which should be seriously taken into consideration for normal levels of Hbs-$A_2$ and F in a $\beta$-thalassemic heterozygote is the simultaneous presence of iron deficiency But the determination of serum iron eliminates this possibility In case of low serum iron, following the correction of iron deficiency with adequate iron therapy the level of Hb-$A_2$ returns to the ratio observed prior to this trace mineral deficiency [24]

Interestingly the levels of Hbs F and $A_2$ were similar in 3 patients with $\beta$-thalassemia intermedia homozygous for $\beta$ thalassemia with normal levels of Hbs-$A_2$ and F A moderate increase in Hb-F ranging between 10 and 15% and a striking elevation in Hb-$A_2$ were recorded in these $\beta$-thalassemic individuals. On the other hand, the results of hemoglobin analyses found in 5 patients with $\beta$ thalassemia intermedia heterozygous for both $\beta$-thalassemia with increased Hb-$A_2$ and $\beta$-thalassemia with normal levels of Hbs-$A_2$ and F differed a little from those observed in homozygous state. The increase in Hb-F was more pronounced, ranging between 11 and 30%.

According to the moderate course observed in these 8 patients, it seems probable that these syndromes are comparatively milder than homozygous $\beta$-thalassemia with increased Hb-$A_2$. Another interesting point is that in these $\beta$-thalassemia syndromes, the level of Hb-F is comparitively low Therefore in cases of thalassemia intermedia associated with slightly or moderately elevated Hb-F this possibility should be considered. On the other hand, in contrast to the patients presented here and the one of BERNINI *et al.* [9] there are some papers describing severe clinical and hematological pictures of this $\beta$-thalassemia syndrome. SHETTINI and MELONI [20] reported a severe case of Cooley's anemia associated with a

high level of Hb-F. Both of the thalassemic parents had normal levels of Hb-$A_2$ and F. Similarly the present authors described a severe case of Cooley's anemia with high amount of Hb-F [5]. One of the parents showed normal levels of Hb-$A_2$ and F. The difference concerning the severity of clinical and hematological pictures and the level of Hb-F of the patients presented here and those of SHETTINI and MELONI [20] and AKSOY and ERDEM [5] suggests the presence of different thalassemia genes responsible for these two groups. Considering the high amount of Hb-F in the latter we are inclined to accept that SHETTINI and MELONI's case was possibly heterozygous for both $\beta$- and $\delta\beta$-thalassemia with normal levels of Hb-$A_2$ and F and the case of present authors reported previously heterozygous for both $\beta$-thalassemia with increased Hb-$A_2$ and $\delta\beta$-thalassemia with levels of Hb-$A_2$ and F.

Formerly the presence of hypothetical 'silent' $\beta$-thalassemia genes expressed as 'thalassemia-like gene which is connected with the formation of human hemoglobin' or 'a thalassemia-like gene which is responsible for $\beta$-chain abnormality' was forwarded by one of the present authors (M. A.) in interpreting an unusual form of thalassemia syndromes [2, 6]. Furthermore, in 1969 SCHWARZ [19] accepted the presence of a 'silent $\beta$-thalassemia gene in one of the normal appearing parent of a patient with $\beta$-thalassemia intermedia with slightly elevated Hb-F following measurement of a marked deficiency in $\alpha/\beta$-chain synthesis.

$\beta$-Thalassemia intermedia heterozygous for both $\beta$-thalassemia with increased Hb-$A_2$ and 'silent' $\beta$-thalassemia can be divided into two subtypes, according to the level of Hb-F: (1) with high Hb-F and (2) with slightly elevated Hb-F. 2 of our patients (cases 18 and 19) can be included in the first subtype as well as some cases with similar $\beta$-thalassemia syndrome reported in the Italian medical literature [21] and one of the cases of homozygous $\beta$-thalassemia of mild variety described by HELLER *et al.* [14]. On the other hand, the last patient (case 20) represents the second subtype. It seems that the case reported previously by one of the present authors (M. A.) as an example of double heterozygosity for $\alpha$-$\beta$-thalassemia also falls into the second subtype [1], because, as PEARSON [18] showed, the combination of $\alpha$-$\beta$-thalassemia has clinical and hematological findings no more severe than in either trait alone [2]. Cases reported by SCHWARTZ [19] are also examples of the second subtype.

The findings obtained in these few families with two types of 'silent' $\beta$-thalassemia must be substantiated by further data from other parts of the world.

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# Erratum

In the article entitled 'Cytogenetic Studies in Acute Leukaemias. Prognostic Implications of Chromosome Imbalances' by G. ALIMENA, L. ANNINO, P. BALESTRAZZI, A. MONTUORO and B. DALLAPICCOLA, published in Vol. 58, No. 4, pp. 234–239, 1977 three tables mentioned in the text were not published.

*Table I* Chromosome analysis according to diagnosis and tissue examined

| Diagnosis | Tissue examined | | | Total number of preparations |
|---|---|---|---|---|
| | bone marrow | peripheral blood | both tissues | |
| ALL | 3 | 2 | 2 | 5 |
| ANLL | 30 | 33 | 15 | 63 |
| Total | 33 | 35 | 17 | 68 |

ALL = Acute lymphoid leukaemia, ANLL = acute non-lymphoid leukaemia.

*Table II* Acute leukaemia patients with chromosome abnormalities on bone marrow samples

| Patient | Diagnosis | Abnormal cells, % | Karyotypes | Clinical course |
|---|---|---|---|---|
| 1 | ALL | 10 | 46, XX<br>47 XX, +21 | full remission |
| | ALL | 7.5 | 46, XY<br>44 XY −21 −14 | full remission |
| 3 | ALL | 25 | 46, XY<br>45 XY −14<br>45 XY −17<br>44 XY −17 −21 | deceased before remission |
| 4 | AML | 7 | 46, XX<br>46, XX, −7 +19 | full remission |
| 5 | AML | 11 | 46, XX<br>47 XX, +8 | full remission |
| 6 | AMML | 5 | 46, XY<br>47 XY +17 | deceased before remission |

*Table II* (continued)

| Patient | Diagnosis | Abnormal cells, % | Karyotypes | Clinical course |
|---|---|---|---|---|
| 7 | AML | 90 | 46, XY<br>50–52, XY +9 +14 +17 +19 +20, +21 | deceased before remission |
| 8 | AML | 100 | 45, X−Y<br>46, X, −Y +8 | partial remission |
| 9 | AMML | 90 | 46, XY<br>46, XY −17 +21<br>47 XY +21 | deceased before remission |
| 10 | AML | 100 | 46, XX, t (5/11)<br>46, XX, t (5/11), 4q+<br>45 XX, t (5/11), −21 | deceased before remission |
| 11 | AML | 60 | 46, XY<br>45, XY −8 −10, +21<br>47 XY +21 | deceased before remission |
| 12 | AML | 73 | 46, XX<br>46, XX, −22, +14<br>46, XX, −7 −20, +14 +16<br>46, XX, −7 −22, +14, +16 | full remission |
| 13 | AMML | 33 | 46, XY<br>47 XY +8<br>48 XY +8, +10<br>48, XY +21 +22 | deceased before remission |
| 14 | AMML | 5 | 46, XY<br>47 XY +8 | full remission |
| 15 | EL | 100 | 47 XY +8 | deceased before remission |
| 16 | EL | 100 | 34–46 chromosomes with multiple structural rearrangements and rings | deceased before remission |
| 17 | EL | 90 | 46, XY<br>40–45 chromosomes with multiple structural rearrangements and rings | deceased before remission |
| 18 | EL | 100 | 44–45 chromosomes with multiple structural rearrangements, marker chromosomes, dicentrics and rings in 35% of cells | deceased before remission |

# Erratum

In the article entitled 'Cytogenetic Studies in Acute Leukaemias. Prognostic Implications of Chromosome Imbalances' by G. Alimena, L. Annino, P. Balestrazzi, A. Montuoro and B. Dallapiccola, published in Vol. 58, No. 4 pp. 234–239, 1977 three tables mentioned in the text were not published.

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| ALL | 3 | 2 | – | 5 |
| ANLL | 30 | 33 | 15 | 63 |
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ALL = Acute lymphoid leukaemia, ANLL = acute non-lymphoid leukaemia.

*Table II* Acute leukaemia patients with chromosome abnormalities on bone marrow samples

| Patient | Diagnosis | Abnormal cells, % | Karyotypes | Clinical course |
|---|---|---|---|---|
| 1 | ALL | 10 | 46, XX<br>47 XX, +21 | full remission |
| 2 | ALL | 7.5 | 46, XY<br>44 XY −21 −14 | full remission |
| 3 | ALL | .5 | 46, XY<br>45 XY −14<br>45, XY −17<br>44 XY −17 −21 | deceased before remission |
| 4 | AML | 7 | 46, XX<br>46, XX, −7 +19 | full remission |
| 5 | AML | 11 | 46, XX<br>47 XX, +8 | full remission |
| 6 | AMML | 5 | 46, XY<br>47 XY +17 | deceased before remission |

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# Intracerebral Calcifications in Childhood Lymphoblastic Leukemia

## A New Iatrogenic Disease?

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*Key Words.* Acute lymphatic leukemia · Calcification · CNS involvement in leukemia · Irradiation · Methotrexate

*Abstract.* A 3-year-old child diagnosed as having acute lymphatic leukemia (ALL), developed meningeal leukemia 36 months after the onset of the disease. H was twice subjected to cranial irradiation plus intrathecal methotrexate (i.t. MTX). Skull radiology showed bilateral gyriform calcification of both cerebral hemispheres. Hematological relapse was first detected 5 years after diagnosis and the child died 5 months later.

The most striking findings of right frontal lobe biopsy and the postmortem examination were wide calcium deposits located in the cortex and in the adjacent white matter. Intense demyelination as well as areas of neuron poverty were apparent in the necropsy but not in the biopsy specimen. The possible interrelationship between such deposition and cranial irradiation and/or i.t. MTX suggests a new iatrogenic disorder.

Central nervous system (CNS) involvement is a usual event in the natural history of acute lymphocytic leukemia (ALL) [1]. The leukemic cell infiltrates (meningeal leukemia) and thrombocytopenic hemorrhages can be regarded as the classical clinicopathological forms of such involvement, although their frequency has been dramatically reduced by the use of CNS prophylaxis and of platelet concentrates. On the other hand, infection, arachnoiditis, direct lesions to the spinal cord, or demyelinating leukoencephalopathy might be found with increasingly frequency, proba-

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*Table II* (continued)

| Patient | Diagnosis | Abnormal cells, % | Karyotypes | Clinical course |
|---|---|---|---|---|
| 19 | EL | 100 | 45 XY −4 −21 −22, +16, +R<br>47 XY −8, −21 +16, +R +M<br>XY −8 −21 −22, +14, +2R, +M<br>XY −21 −22, +14 +16, +R<br>XY −7 −8 −10, −21 +14 +16, +2R, +M<br>XY −7 −21 +16, +R, +M<br>48 XY −7 −8, −21 +14, +16, +2R, +M<br>49 XY −7 −8, +16, +16, +2R, +M | deceased before remission |

ALL = Acute lymphoid leukaemia, AML = acute myelocytic leukaemia, AMML = acute myelomonocytic leukaemia, EL = erythroleukaemia, R = ring, M = marker

*Table III* Chromosome analysis of peripheral blood cultured leukocytes

| Diagnosis | Examined cases | Cases with abnormal cells | Abnormal karyotypes |
|---|---|---|---|
| ALL | 2 | 1 | 46, XX (12% of cells with chromosome instability Louis-Bar syndrome) |
| ANLL | 33 | 5 | 47 XX, +3 (2 cells)<br>47 XX, +14 (1 cell)<br>47 XY +8 (3 cells)<br>47 XY +marker (4 cells)/47 XY +8 (1 cell)/ 47 XY +ace (3 cells)<br>47 XY +ace (D-like 1 cell) |
| Total | 35 | 6 | |

ALL = Acute lymphoid leukaemia, ANLL = acute non-lymphoid leukaemia

both high cerebral functions and coordino-equilibration but there no association of extrapyramidal signs. A right frontal lobe biopsy was performed 1 month later

Hematological relapse was detected for the first time in February 1975, along with 450 blast cells/mm³ in CSF. The disappearance of the latter was achieved with i.t. MTX, but hematological remission failed to occur despite the administration of VCR, daunomycin (DMC) and PRD for 45 days, followed by cytosine arabinoside, DMC and PRD discontinuously. An evident increase in calcification was detected by skull radiology in May 1975 (fig 1), with pattern of lesions, although bilateral, resembling those of the Sturge-Weber syndrome. The patient neurological status proceeded to worsening progression of apathy and sleepiness accompanied by deterioration of higher cerebral activities, generalized hypotonia, tetraparesis, chiefly appreciated in the lower limbs, and bilateral amaurosis (which was difficult to evaluate due to retinal hemorrhages). He died in October 1975

## *Pathology*

### *Right Frontal Lobe Biopsy*

The specimen was totally embedded in paraffin for light microscope observation. HE, PAS, Rio-Hortega, Luxol-fast blue, Von Kossa, Holzer and Perls stains and reactions were performed.

In the grey matter normal cell population was present, with a slight pericellular edema. Basophilic bodies, concentrically arranged were identified in the basal areas of the cortex by Von Kossa's reaction these complexes were found to be calcium (fig 2). Slight spongy degeneration, as well as focally distributed astrogliosis, appeared in the white matter

### *Necropsy*

2 h postmortem the brain gyri appeared somewhat flattened and discrete vascular congestion was found. A crackling sound was produced by the knife as coronal and transversal sections were made, its intensity increasing in the posterior areas. This sound resulted from the presence of hard, whitish granules, minged with hemorrhagic foci which, on the frontal lobes, were more abundant in the right hemisphere. In the parietal, temporal and occipital lobes the left hemisphere contained more such granules. Interhemispheric fissure appeared deviated to the left, showing a moderate atrophy of the left hemisphere similarly dilatation of the lateral ventricles, mainly the left one was found (fig. 3).

### *Microscopy*

Different samples of brain and of the three portions of the spinal cord were taken for examination under light microscope the staining procedures used for the biopsy were then carried out. A section of cerebral tis-

bly due to the more aggressive therapeutical approach these patients are currently subjected to

*A picture of severe neurological derangement and intracerebral calcifications* resembling those seen in the Sturge Weber syndrome has recently been reported by different authors [6 10 14] in children with ALL who have received whole brain irradiation (WBI) and/or intrathecal methotrexate (I t MTX) The scarcity of such observations, in spite of the high number of patients presently subjected to prophylaxis of CNS leukemia supports the following report

## *Case Report*

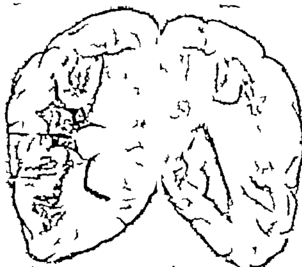
M D C a male born on October 14 1966, was diagnosed as having ALL in December 1969 The family and patient history is not significant Remission induction was started with vincristine (VCR) 2 mg/m² i v weekly and oral prednisone (PRD) mg/kg daily complete remission was attained 1 month later After that time, he received three 5-mg doses of i t. MTX no WBI was then associated. Maintenance therapy was continued with 6-mercaptopurine 75 mg/m²/day orally plus MTX 15 mg/m² twice a week, i.m. every 6 months weekly doses of VCR plus 8 days of oral PRD were given as intensification therapy followed by two 5-mg doses of i t MTX.

In July 1972, persisting in hematological remission, generalized seizures occurred and the patient was admitted to hospital because of postictal coma. Cerebrospinal fluid (CSF) examination was normal and the EEG showed diffuse slowing of background activities mainly in the left parietooccipital area. Anticonvulsants thereafter associated with hematologic therapy attained an important reduction in the frequency of the seizures, although these were not totally suppressed.

In January 1973 with normal bone marrow and peripheral blood, the child presented symptoms suggestive of hypothalamic involvement, namely polyphagia, polydypsia and pathological weight gain Lumbar puncture showed numerous blastic cells in CSF but no neurological deficiency could then be appreciated. Furthermore, skull and sella turcica radiology as well as 17 hydroxy and ketosteroid excretion were normal A pneumoencephalogram showed no significant alterations, and a repeat EEG was similar to the previous ones. The patient received ,400 rad of WBI plus five doses of i.t. MTX 5 mg each.

In May 1974 he was still in hematological remission, but was again admitted to hospital with psychic derangement (his IQ had fallen from 0.90 in May 1971 to 0.70 in May 1974), bradykinesia and listlessness the frequency and severity of seizures increased and the EEG showed evident deterioration. He was again irradiated receiving a dosage to the brain similar to that of the previous occasion

On a pneumoencephalogram, carried out in October 1974 a ventricular enlargement became apparent pl in skull radiology showed subtle ill-defined gyriform opacifications suggesting intracerebral calcification. Clinically the patient, by that time, had a diffuse encephalic syndrome characterized by severe deterioration of



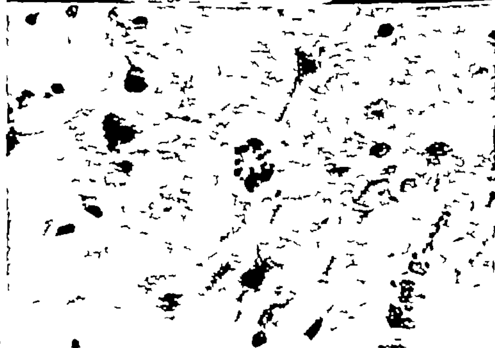
3

4

*Fig 3* Xerograph of brain slice showing bilateral calcifications and expansion of left lateral ventricle

*Fig 4* Postmortem section of brain with numerous widespread calcium deposits and total loss of neurons. HE ×40

1



2

*Fig 1* Gyriform appearance of brain calcification in skull radiography

*Fig 2* Right frontal lobe biopsy Neurons and calcium spherules can be appreciated HE × 100

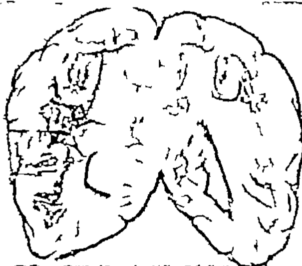


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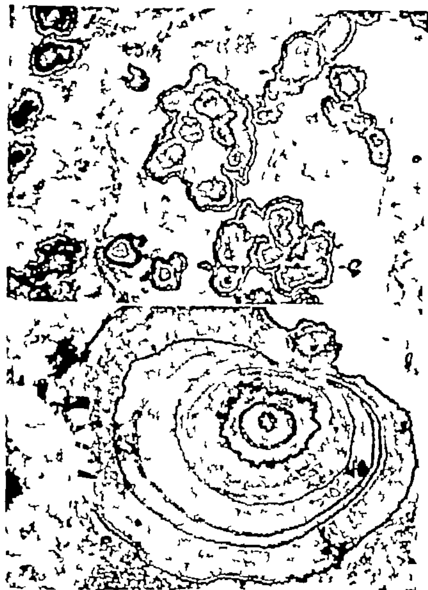



*Fig. 5* Concentric deposition of calcium salts showing no relationship with cells or cell appendages. ×4,000

*Fig. 6* A great basophilic calcium mass located in cerebral tissue. The concentric arrangement of calcium in several layers is evident. ×2,000

sue was fixed and embedded in Epon 812 for examination under a Siemens Elmiskop I A electron microscope

A great number of basophilic masses, which Von Kossa s reaction identified as calcium deposits, could be appreciated in the basal portion of cortex and in the adjacent white matter Loss of neurons was evident, being total in the areas of highest calcium content (fig 4) In these places, a marked glial reaction was found, consisting mainly of astroglia and oligodendroglia. By silver impregnation techniques, the calcium deposits *were found to be located inside the axons, but they also* appeared scattered between the cells and without relationship to neuron appendages (fig 5 6)

An evident spongy degeneration was found in the white matter with demyelination and astro- and oligodendroglial reaction located in wide areas related to calcium deposition (fig. 7) These pathological changes did not appear in those regions close to the unaffected cortex. In the cerebellum the pattern of calcium distribution was similar to that of the cerebrum, while Purkinje s cells were normal No abnormalities could be found in the brain stem

The spinal cord showed a diffuse demyelination in all three portions, although it became more apparent in the descending tracts, except in those originating in the cerebellum Neuron population was normal In the thoracic region leukemic meningeal infiltrates were found in the sensory roots (fig. 8) The cerebral meninges were not affected

Leukemic infiltrates were detected in the bone marrow liver spleen, lymph nodes, kidneys, adrenals, and alimentary canal as well Esophageal and small intestine candidiasis was also apparent

Under electron microscope cerebral structure was greatly disturbed by widespread calcium masses which appeared concentrically arranged and unrelated to the cells (fig 5 6) Neurons showed wide cytoplasms containing scarce mitochondria and some variable-sized vacuolae Lipofuscin-stained granules were apparent, distributed chiefly around nuclei Nuclear retraction along with peripheral distribution of chromatin and prominent nucleoli were also commonly observed

## *Discussion*

Intracerebral calcification is a well known fact in different pathological conditions. In the patient reported here the pattern of calcifica

tion resembled that described in the Sturge-Weber syndrome nevertheless, the lack of a congen tal trait and of facial nevus, as well as the patholog cal findings and also the fact that calcium deposits were bilaterally appre ciated allow that disease to be discarded Intracerebral calcification may appear in different disease states which have been clearly reviewed by BABBIT *et al* [3] but the clinical and roentgenological data found in this patient seemed to correspond to neither of such conditions.

Thus, the singularity of the present report lies upon the spread of calcium deposits, which affected wide cortico-subcortical areas. No calcification was found around the small capillary walls as in the case reported by FLAMENT DURAND *et al.* [10] on the contrary this finding might suggest that calcification takes place in cortical spaces, unrelated to blood vessels but following the boundary between grey matter and white matter and thus achieving the typical gyriform appearance in radiographs.

Striking demyelination and areas of great neuron poverty were apparent in postmortem examination, in open contrast with the biopsy findings. This fact clearly indicates in itself the rapid evolution of such lesions Calcium and demyelination, however showed a close interrelationship in the brain, the areas of greater demyelination being anatomically or functionally dependent on those with a higher calcium content, either in the cortex or in the underlying white matter

From a pathological and clinical standpoint, the data provided by the present case are much in agreement with the findings of BORNS and RANCIER [6] and of FLAMENT-DURAND *et al* [10] The possible relationship between brain calcification and previous antileukemic therapy on CNS is discussed in both reports, as well as in those of GASTAUT and GASTAUT [11] and of KOMP and COX [1.] although the patient reported by these last authors was treated with i.t. MTX, receiving no irradiation to CNS.

MTX toxicity covers a wide spectrum and can be appreciated in different parts of the body [7 17 18] When given intrathecally this drug may induce fever [15] paraplegia [21] arachnoiditis and demyelinating leukoencephalopathy [16 0] and even death [4] The drug s neurotoxicity has been attributed to several mechanisms including pharmacodynamic factors [13] preservatives [5] and antifolic effect [9] this last being specifically exerted on myelin formation [13] However SHAPIRO *et al* [22] on the basis of 15 years experience with i.t. MTX during which scarce neurotoxicity had been observed, expressed their uncertainty about MTX-induced neurological impairment.

The effect of ionizing radiations on CNS has been reviewed by RUBIN-




*Fig 7* Spongy degeneration of wh te matter in brain section along w th glial reaction. HE. 20

*Fig 8* Section of the thoracic region of spin l cord Spongy degeneration as well as meningeal leukemic infiltrates can be seen HE. 20

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STEIN [19] although the likelihood of focal calcifications when these agents are applied is not specially stressed by this author Nevertheless, the vascular changes described by RUBINSTEIN [19] were not apparent in the present case Since the patient received combination therapy with I.t. MTX plus WBI it is difficult to ascribe with certainty the leading role on the determination of calcium deposits to either or both agents. However calcification and leukoencephalopathy appeared to be directly related to CNS therapy as suggested by the lack of previous symptoms and by the patient s normal skull radiographs 1 year before (as similarly reported by FLAMENT DURAND *et al* [10]) On the other hand RUBINSTEIN *et al* [20] and AUR *et al* [2] have pointed to the relationship between irradiation plus MTX and demyelinating leukoencephalopathy specially when the latter agent (MTX) is given intravenously

A further pathophysiological mechanism to explain the neurological derangement observed in some ALL patients has recently been suggested by GASTAUT and GASTAUT [11] who ascribe CNS pathological changes to leukemic cerebral infiltrates. In the present case however such a hypothesis can be excluded since no parenchymatous CNS infiltrates could be found in the pathological examinations

Spongy degeneration plus demyelination comprise the pattern of dysmetabolic encephalopathies as illustrated by VAN BOGAERT [23] in which mucopolysaccharides or other substances may induce calcium precipitation with the catalytic action of Zn ions however in a patient with ALL and brain calcification on whom trace element quantifications were carried out Mn showed the most striking changes [14] Nevertheless, since the metabolism of different metals appears to be impaired in acute leukemia [8] this, along with the concurrence of radiation and i t MTX might possibly account for the deposition of calcium salts on a previously damaged nervous system

One could thus be assisting at the coming of a new sort of iatrogenic pathology which would arise from metabolic and perhaps individual events that at the present remain totally unknown

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Acta haemat. 59 205–214 (1978)

# Sequential Therapy with Daunorubicin and L-Asparaginase in Relapses of Acute Lymphoblastic Leukemia in Children

G. MASERA, C. UDERZO, E. CORBELLA and V. CARNELLI

Department f Pediatrics and Child Health, Milan University Milan

*Key Words* Daunorubicin -Asparaginase Relapse Acut lymphoblastic leukemia Remission Toxic effects

*Abstract.* 13 children affected by acut lymphoblastic leukemia in advanced stage of illness received sequential therapy with daunorubicin and L-asparaginase. During daunorubicin therapy significant decrease f bone hypercellularity as well as circulating cells occurred, further cycle with scarcely myelotoxic drug, L-asparaginase, was administered. 10/13 (76.9 ) remission of the disease was then achieved. This therapeutic trial was well accepted and could be more extensively used in the patients in relapse A remission lasting between 4 and 28 weeks was observed

## *Introduction*

L-Asparaginase is an enzyme with antiblastic activity it produces remission in about 30 of patients with acute lymphoblastic leukemia (ALL) and is most effective in combination therapy [3 6, 10, 12, 13 15]

Daunorubicin has a marked antitumor activity (20–55 / of complete remissions in acute leukemias) and independent toxicity from L-asparaginase [2–7 14] On this basis and considering the results of MC ELWAIN and HARDISTY [9] and BODEY *et al* [1] the effectiveness of a sequential therapy consisting of an initial administration of daunorubicin followed by a cycle of L-asparaginase has been evaluated.

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## Case Reports and Methods

13 children between 3 and 8 years of age, affected by ALL, were entered on the study. At the beginning of their illness they had all been treated with an 'attack' regime of vincristine, prednisone and a prophylaxis of central nervous system with methotrexate intrathecally. When complete remission was attained, a 3-month maintenance therapy followed. It consisted of mercaptopurin and methotrexate alternated with a 15-day cyclic regime of vincristine and prednisone. All the patients had one or more relapses during these stages of illness they had all been treated with daunorubicin at a median dose of 12.6 mg/kg (table I). 3 of them had been previously treated with prednisone and L-asparaginase (at a median dose of 2,633 U/kg) and a single child had undergone a previous successful trial with daunorubicin and L-asparaginase (case 6b, table I).

The median duration of survival from the beginning of our observation was 21 months. At this point we have introduced a sequential therapy as follows. Prednisone 2 mg/kg/day orally in three doses for 3–4 weeks, daunorubicin 1 mg/kg/day i.v. for 3 or 4 days. After this period, if a high number of blasts was still in the peripheral blood, a second course of therapy was instituted with daunorubicin 4 days after the first trial. A day after the last dose of daunorubicin, L-asparaginase at a dose of 200 U/kg/day was administered until a complete remission was attained or in a case of failure or partial remission, for 23–28 days. In a single case (No. 13) the administration of L-asparaginase was discontinued because of an allergic reaction towards the antitumor agent (the patient was in partial remission). Serial bone marrow biopsies were performed before therapy, after daunorubicin and after L-asparaginase; our criteria for bone marrow remission are those defined by the St. Jude group [11]. Peripheral blood cell counts were determined at least three times weekly during the remission induction period. Studies of cardiac, hepatic and renal function were performed at intervals along with routine determinations. Patients in complete remission were maintained with methotrexate (20 mg/m² i.m.) twice a week for 2 months and then with mercaptopurin (2.5 mg/kg/day orally).

## Results

The results in our patients (all of them had been previously treated with daunorubicin) are summarized in table I. Out of 13 patients treated 10 obtained complete remission (76.9%) and 3 partial remission. In case No. 6 a preliminary tentative therapy led to a complete remission. The same patient, who relapsed after 5 weeks, showed a good response to daunorubicin, while he showed signs of a new relapse during treatment with L-asparaginase. In 3 cases (No. 1, 2, 12) already previously treated with prednisone and L-asparaginase, our scheme was effective in inducing a complete remission in all of them. Figure 1 illustrates the marrow as

*Table I*

| Case No | Age, years | Sex | Duration of illness months | Relapse | Drugs previously administered in acute phases daunorubicin total dose, mg/kg L-asparaginase total dose, U/kg | Days of treatment with daunorubicin 1 mg/kg/day | Days of treatment with L-asparaginase 200 U/kg/day | Result | Duration of remission weeks |
|---|---|---|---|---|---|---|---|---|---|
| 1 BA | 4 | M | 32 | 7th | prednisone<br>vincristine<br>daunorubicin (19)<br>L-asparaginase (2,000) | 4 | 28 | complete remission | 7 |
| 2 CM | 7 | M | 21 | 4th | prednisone<br>vincristine<br>daunorubicin (18)<br>methotrexate | 7 | 1 | complete remission | 7 |
| 3 AA | 4 | F | 15 | 1st | prednisone<br>vincristine<br>daunorubicin (8) | 3 | 3 | partial remission | |
| 4 FE | 8 | F | 46 | 3rd | mercaptopurine<br>prednisone<br>daunorubicin (10)<br>vincristine | 3 | 10 | complete remission | 8 |

*Table II* Toxic effect attributed to single drugs

| Case No. | Cardio-toxicity | Alterations in the mucous membranes | Alopecia | Myelo-toxicity | Allergic reactions[1] | Reduction in blood levels of[3] fibrinogen mg/dl | albumin g/dl | cholesterol mg/dl | SGOT SGPT increased[4] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | – | – | – | – | nettle rush | 110 | 4 | – | |
| 2 | – | – | – | – | – | 100 | 4 | – | |
| 3 | – | – | + | – | – | | – | – | |
| 4 | – | – | – | – | anaphylactic shock | – | – | – | |
| 5 | – | – | – | – | anaphylactic reactions | – | 3,5 | 150 | |
| 6a | – | – | – | – | – | – | 3,5 | 160 | 105/70 |
| 6b | – | – | – | +[2] | – | – | 3,5 | – | 110/75 |
| 7 | – | – | – | – | – | – | – | – | – |
| 8 | – | + | – | +[2] | – | | 3,3 | 180 | 95/49 |
| 9 | – | – | – | – | moderate anaphylactic reaction | – | | – | |
| 10 | – | – | – | – | | | | – | 80/40 |
| 11 | – | – | – | +[1] | | 130 | | 190 | 100/50 |
| 12 | – | – | – | +[2] | | – | | – | |
| 13 | – | – | – | – | anaphylactic reaction | | | | |

[1] With L-asparaginase.
[2] With daunorubicin.
[3] Minimal value.
[4] Maximal value/U

pect of 12 patients in various stages of treatment. The data relating to the case number 8 are not reported, as the marrow specimen obtained after daunorubicin treatment showed a marked hypoplasia. It can easily be observed that daunorubicin induced a marked reduction in the blastic quota in only 2 cases (5 and 9). In nearly all the patients the number of white blood cells at the end of the cycle with daunorubicin was found to lie between 600 and 3 000 mm³ (fig. 2). This leukopenia did not hinder progression to the second stage of treatment with L-asparaginase. We encountered a moderate thrombocytopenia quite often, but it was not severe enough to require supplementation with a platelet concentrate. Finally the remissions obtained were usually of short duration, varying from a mini

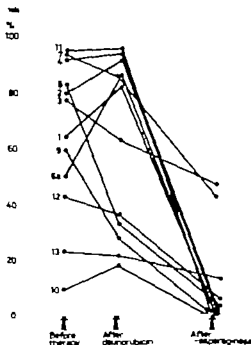



*Fig. 1.* Blasts in the bone marrow before and after therapy

mum of 4 to a maximum of 28 weeks (medium duration of 10 weeks, in 8 of 10 cases shorter than 9 weeks).

*Tolerability*

The most significant toxic effects in our group of cases are reported in table II. Daunorubicin caused no signs of cardiotoxicity (according to the electrocardiographic control and creatinphosphokinase determination) or important alterations in the mucous membranes. Only in 3 cases its myelotoxic action was particularly marked (cases 6b, 8, 12), while in another case [11] a severe marrow aplasia became evident in the 2nd week of treatment with L-asparaginase.

In the second stage of the scheme, 5 patients showed hyperergic-allergic reactions, which culminated in fairly marked anaphylactic shock in cases 4 and 5. Always in this stage we frequently noted moderate signs of hepatic damage such as increase of transaminases, decrease in blood lev-

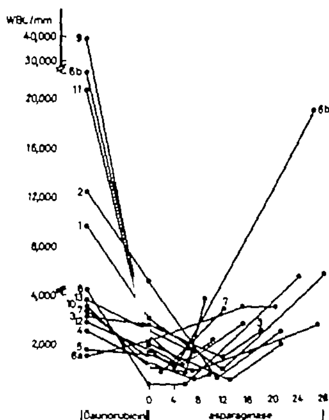



*Fig 2* Lowest value of white cells during therapy with L-asparaginase

els of albumin fibrinogen and other clotting factors synthesized in the liver but all these parameters returned rapidly to normal and no bleeding was observed

## *Discussion*

With our therapeutic schedule it was necessary to carry out the treatment in two distinct phases. In the first we were trying to obtain a rapid, marked destruction of the leukemic cells using daunorubicin which inhibits DNA-dependent RNA synthesis [4 5 8]

Therapy was then continued with L asparaginase an equally effective drug which allowed us to achieve remission without serious risks of marrow aplasia Previous treatment with daunorubicin might potentiate the action of L asparaginase in two possible ways. (a) by synchronizing the

mitotic cycle of the leukemic cells and making them more sensitive to the second drug, (b) by causing a further decrease in the marrow cells that could supply L-asparagina to the blastic compartment [9].

Our results show the effectiveness of such a therapeutic schedule: complete remission was reached in 76.9% of subjects, a higher percentage than that obtained with single drugs. Our clinical observations seem to confirm therefore the experimental data which demonstrate that the effectiveness of L asparaginase is increased when used sequentially with daunorubicin.

More recently BODEY *et al.* [1] reported their own experience in treating adult acute leukemia with daunorubicin and L-asparaginase but with different administration schedules and results we cannot easily compare.

However our experience can be summarized as follows. (1) Good results have been obtained for patients already resistant to the conventional antiblastic drugs, all of them previously having been treated with daunorubicin and 3 with both prednisone and L-asparaginase. (2) L Asparaginase played a prominent role in inducing remission in 2 of 13 patients: as a matter of fact in 2 cases (1 and 6a, fig. 1) in which daunorubicin did not affect either bone marrow or peripheral blood, a subsequent therapy with L-asparaginase determined a prompt disappearance of blasts and a complete remission. (3) The administration of two drugs at a short interval has usually been well tolerated.

Finally according to MC ELWAYN and HARDISTY [9] although the duration of the remission has been short, the use of L-asparaginase in schemes of sequential therapy in combination with either daunorubicin or adriamycin or cytosine-arabinoside is undoubtedly interesting and it is to be expected that better results can be obtained on the basis of an increased number of clinical trials.

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Acta haemat. 59: 215–222 (1978)

# Cryoimmunoglobulinemia in Four Sisters[1]

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*Key Words.* Cryoglobulin · Immune complexes · Rheumatoid factor · Amino acid analysis · Familial trait

*Abstract.* Mixed-type cryoproteins, consisting of IgG and IgM, were demonstrated in the sera of four sisters. While the IgG component was polyclonal in every instance, in two of them the IgM component was found to be monoclonal with type κ light chains. Clinical diagnoses included the purpura-weakness-arthralgias syndrome, posthepatitic cirrhosis, congestive heart failure and mitral stenosis. The cryocrit differed in the four sisters, ranging from 3 to 16%; in addition, rheumatoid factor activity was consistently associated with both washed cryoprecipitates and their isolated IgM components. Endomembraneous deposits of IgG and IgM were revealed by immunofluorescent studies of the renal biopsy specimen from one patient. A genetic abnormality, possibly of the autosomal recessive type, is suggested in this instance of familial cryoglobulinemia.

Over the past 15 years cold precipitable proteins have been paid increasing attention. A number of immunochemical studies have clearly established that cryoglobulins consist of immunoglobulins [8, 11] and that they may be classified into at least three different groups, namely single type monoclonal cryoglobulins, mixed cryoglobulins with a monoclonal component, and polyclonal cryoglobulins [4, 6]. Despite intensive physicochemical and structural investigations [10, 12] the actual mechanisms of cryoprecipitation have so far escaped elucidation, and it seems likely that along with possible abnormalities of amino acid composition, other

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factors related to the tertiary and quaternary structures (such as hydrophobic and electrostatic bonds) may be involved in the occurrence of the phenomenon.

Whether and to what extent genetic and/or environmental factors may play a role in the induction of cryoglobulinemia, remains largely unknown. It seemed therefore of interest to describe a family consisting of four sisters all of whom had circulating cryoimmunoglobulins.

## *Family Report*

Patient *Bu.L.* is a 59-year-old housewife, who was first admitted to the General Hospital of Piacenza in February 1973 for evaluation of purpura and recurrent leg ulcers, arthralgias, and epigastric discomfort. Past history included appendicectomy in 1947 and duodenal ulcer in 1970. 4 years prior to admission her blood pressure was found to be elevated. On physical examination the skin of the buttocks and lower extremities showed petechial lesions at different stages of evolution. A mild edema of both ankles was also present.

Laboratory findings included persistent glomerular-type proteinuria, significant (7–13 / ) eosinophilia on the peripheral blood smears as well as in the bone marrow and high serum levels of anti-$\gamma$-globulin antibodies as measured by the latex slide test and the tanned sheep cells agglutination test. Platelet counts ranged between 166,000 and 172,000 platelets/mm³. Measurement of some serum complement components gave the following values: C3c 51 mg/dl, C4 6 mg/dl and C3PA 13.5 mg/dl. A remarkable cryoprecipitation occurred in her serum after storage at 4 °C for 2–3 days.

Despite protection from cold and administration of prednisone (20 mg/die) and azathioprine, the clinical course was characterized by recurrences of arthralgias and purpuric eruptions on both legs. When last seen (June 1977), physical examination and laboratory data were mostly unchanged.

*Bu.T.* a 71-year-old housewife, was referred to the 2nd medical department General Hospital of Piacenza, for evaluation of progressive abdominal distention, oliguria, and pitching edema of both legs. At age 50 she had fever and icterus of 4-months duration, which were ascribed to viral hepatitis. One year later cholelithiasis was diagnosed and a cholecystectomy was performed during surgery the liver was reported as showing a sclerotic appearance. Since then, the patient has been repeatedly hospitalized because of abdominal distention, lowered urine output and dyspepsia. In 1975, a bout of hematemesis and melena occurred requiring the insertion of the Blakemore-Sengstaken tube to tamponade the esophageal varices.

On admission, the liver edge was felt four fingerbreadths below the right costal margin and the spleen tip three fingerbreadths below the left costal margin. Additional physical signs included moderate ascites, scleral icterus, and petechiae of the lower extremities. Among routine laboratory data, liver function tests were consistently impaired, and platelet counts revealed abnormally low values ranging from

25,000 to 42,000 platelets/mm While hepatitis-B surface antigen was absent in the patient's serum on several occasions, detectable levels of anti-HB antibodies were persistently found. A cryoprecipitate appeared during 96 hour incubation of a serum sampl at 4 °C.

*Bu.G* who is now 64, had no significant illnesses until the age of 30 years, when she sought medical attention complaining of sore throat, fever and joint involvement. Acute rheumatic fever was diagnosed, and penicillin therapy was started. 2 years later she developed dyspnea on exertion, fatigue, and palpitation. Assessment of cardiovascular function allowed diagnosis of mitral stenosis. 7 years ago valve replacement with prosthetic device was performed. Because of atrial fibrillation and heart failure, she is being treated with cardiac glycosides and diuretics.

*B M* 76-year-old woman, complains of vertigo, tinnitus, and headaches. Both systolic nd diastolic blood pressures hav been consistently found to be moderately elevated in the last tw years. A good, though labile hemodynamic compensation is usually obtained by administration of digitalis and diuretics. Past history is unremarkable.

There were no further siblings in family *B* Both parents lived to be quite elderly and in none of the children of the four affected sisters could cryoprecipitation be demonstrated.

## *Special Studies*

The techniques of blood collection, isolation and purification of cryoglobulins and the methods applied for immunochemical studies of the isolated components have been described in previous papers [3, 6]

Analytical ultracentrifugation was carried out in Beckman Spinco Model E ultracentrifuge at 59 780 rpm and 37 C. Separation of each component from mixed cryoglobulins was achieved by gel filtration of the purified cryoprecipitates on Sephadex G-200 columns, using an Na-acetate buffer 0 2 M, pH 4.0.

For amino acid analyses, protein samples were hydrolyzed for 22 h with 6 N HCl at 110 °C in an evacuated sealed tube and then analyzed with the Beckman Model 120 C automatic amino acid analyzer equipped with high sensitivity cuvets and recorder

Morphologic studies were carried out by light microscopy on tissue fragments obtained during renal biopsy After f xation in Bouin's solution, 4 µm-thick sections ere cut and stained by standard histologic methods. For immunofluorescence, tissue samples were frozen in liquid nitrogen and cut at 3-5 µm in cryostat at 20 C The sections were fixed in acetone and then stained with fluorescein-labeled antisera directed against human IgG IgA, IgM, C3 and fibrinogen (Hoechst Behringwerke, Marburg Lahn, Germany)

## *Results*

During a 96-hour incubation at + 4 C a cryoprecipitate appeared in each of the serum samples from the four sisters. Cryocrit estimation, eval

factors related to the tertiary and quaternary structures (such as hydrophobic and electrostatic bonds) may be involved in the occurrence of the phenomenon

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## *Family Report*

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Laboratory findings included persistent glomerular type proteinuria, significant (7–13 / ) eosinophilia on the peripheral blood smears as well as in the bone marrow and high serum levels of anti-$\gamma$-globulin antibodies as measured by the latex slide test and the tanned sheep cells agglutination test Platelet counts ranged between 166,000 and 172,000 platelets/mm$^3$ Measurement of some serum complement components gave the following values: C3c 51 mg/dl, C4 6 mg/dl and C3PA 13.5 mg/dl A remarkable cryoprecipitation occurred in her serum after storage at 4 C for ~3 days.

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On admission, the liver edge wa felt four fingerbreadths below the right costal margin and the spleen tip three fingerbreadths below the left costal margin Additional physical signs included moderate ascites, scleral icterus, and petechiae of the lower extremities. Among routine laboratory data, liver function test were consistently impaired, and platelet counts revealed abnormally low values ranging from

*Table I* Quantitative estimation and immunochemical characteristics of the serum cryoprecipitates in four sisters

| Subject | Cryocrit % | IgG component L-chain type | IgG component RF activity | IgM component L-chain type | IgM component RF activity |
|---|---|---|---|---|---|
| Bu. E. | 16 | + λ | – | κ | + |
| Bu. T | 9 | + λ | – | κ | + |
| Bu. G | 4 | κ + λ | – | κ + λ | + |
| Bu. M | 3 | κ + λ | – | + λ | + |

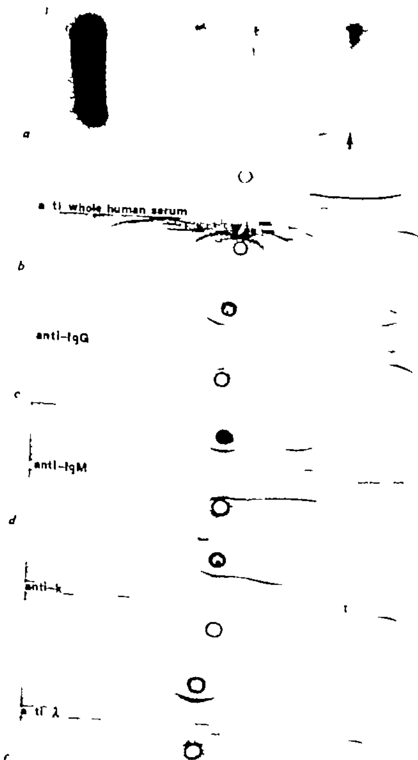
RF = Rheumatoid factor
Evaluated by centrifugation of sera in Wintrobe tubes (200 g for 20 min at + 4 °C).

uated by centrifugation at 200 g for 20 min in a cold room, revealed remarkable variations from case to case in the amount of the cold-precipitable material (table I). Immunochemical studies showed all four cryoprecipitates to be IgM IgG mixed cryoglobulins. In addition while the cryoglobulins isolated from the sera of Bu.G and Bu.M. were heterogeneous immunoglobulin molecules in that both IgG and IgM components included Λ and λ-chains, in the remaining two cases the IgM component was monoclonal (fig. 1) It should also be mentioned that rheumatoid factor activity was consistently associated with the purified cryoproteins and their isolated IgM components.

Owing to the relatively low cryocrit in the remaining sera, amino acid analysis was performed with the cryoglobulin from serum Bu.E. only Table II gi es the amino acid composition of the isolated μ-chains and κ-chains from the IgM monoclonal component of such cryoglobulin. No grossly evident aberrations could be shown as regards the amino acid composition of the tested material

Light microscopic study of the renal biopsy specimens from patient Bu.E. suggested membranoproliferative glomerulonephritis with mesangial cell proliferation and capillary wall thickening. Capillary loops often

*Fig. 1* Cellulose acetate electrophoresis of the serum from patient Bu.E. The arrow points to the serum M-component. *b–f* Immunoelectrophoresis of the purified cryoprecipitat from the same patient (upper w ll) and of pooled normal human serum (lower well). Note that both IgG and IgM are present in such cryoglobulins and that the IgM component includes light chains only


a
anti whole human serum
b
anti-IgG
c
anti-IgM
d
anti-k
anti λ
f

single-type monoclonal cryoglobulins or mixed cryoglobulins with an IgM monoclonal component. Cryoprecipitation occurs in a significant number of sera from patients with infectious diseases associated with autoimmune features [3 9] connective tissue diseases [14 15] and lymphoproliferative disorders [4 6] In addition a symptom complex including purpura-weakness-arthralgias, which is thought to represent a distinct clinical entity has been frequently observed in association with cryoglobulinemia [4 6, 11] Approximately one-third of such patients develop a severe and rapidly progressive form of renal disease [1 5] Cold precipitates have also been described in a group of patients with acute and chronic liver diseases [7]

The 4 cases reported in this paper were no exception as regards their clinical evaluation. Indeed patient Bu.E. had membranoproliferative glomerulonephritis with progressive renal failure complicating primary mixed cryoglobulinemia, whereas posthepatitis cirrhosis was diagnosed in patient Bu.T The remaining 2 cases (Bu.M. and Bu.G ), exhibiting lower amounts of circulating cold-precipitable proteins, had congestive heart failure and mitral stenosis, respectively

In view of the consistent rheumatoid factor-activity of the purified cryoglobulins and their isolated IgM components, the role of such mixed cryoglobulins as antigen-antibody complexes of the IgG/anti-IgG type can no longer be doubted.

The occurrence of cryoglobulinemia in four sisters may hardly be ascribed to coincidence. To our knowledge, relatively few observations of familial cryoglobulinemia have been published so far affecting two brothers [2] or the proband and two sons [13] Although no clear-cut explanation may be given, it seems likely that genetic and/or environmental and or infectious factors may play a role in the pathogenesis of the phenomenon.

While a genetic abnormality may be suggested in family Bu., no definite conclusions can be drawn regarding the mode of inheritance of the defect leading to the protein changes. An autosomal recessive trait seems, however to be the most likely explanation.

## *References*

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*Table II* Amino acid composition (average of three analyses) of the IgM/κ monoclonal component isolated from the mixed cryoglobulin of patient Bu. E.

| Amino acid[1] | mol residues/mol protein | |
|---|---|---|
| | μ-chain | κ-chain |
| Lysine | 41.5 | 15.0 |
| Histidine | 8.7 | 1 7 |
| Arginine | 17 4 | 10 0 |
| Cysteic acid | 14 0 | 2.5 |
| Aspartic acid | 6[illegible]9 | 20 0 |
| Threonine | [illegible]7 | 15 0 |
| Serine | 60 0 | 33.5 |
| Glutamic acid | 64.2 | — |
| Proline | 40.3 | 10 0 |
| Glycine | 45 4 | 15.0 |
| Alanine | 41 8 | 17.5 |
| Valine | 40.8 | 14 0 |
| Methionine | 10.0 | 2.[illegible] |
| Isoleucine | 10.9 | 7.2 |
| Leucine | 44 6 | 7.5 |
| Tyrosine | 16.0 | 9.0 |
| Phenylalanine | 19 0 | 10.1 |
| | 570.[illegible] | 21[illegible]4 |

[1] Tryptophan was not calculated.

appeared to contain PAS-positive thrombi A moderate degree of tubular atrophy was also observed. Immunofluorescent studies of the endomembraneous deposits showed positive staining with anti-IgG and anti IgM sera appearing as a granular deposition along the basement membrane in subendothelial position Positive results, although to a much lower extent, were obtained with a fluoresceinated anti-C3

## *Discussion*

Screening of large numbers of refrigerated sera has clearly shown that spontaneous precipitates which dissolve upon heating at 37 C are not infrequently recognized Although the cryoglobulin concentration is usually rather low and appears as a whitish precipitate at the bottom of the tube much higher amounts may be found in a few cases, usually consisting of

single-type monoclonal cryoglobulins or mixed cryoglobulins with an IgM monoclonal component. Cryoprecipitation occurs in a significant number of sera from patients with infectious diseases associated with autoimmune features [3 9] connective tissue diseases [14 15] and lymphoproliferative disorders [4 6] In addition, a symptom complex including purpura-weakness-arthralgias, which is thought to represent a distinct clinical entity has been frequently observed in association with cryoglobulinemia [4 6, 11] Approximately one-third of such patients develop a severe and rapidly progressive form of renal disease [1 5] Cold precipitates have also been described in a group of patients with acute and chronic liver diseases [7]

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| Cysteic acid | 14 0 | 2 5 |
| Aspartic acid | 6[illegible].9 | [illegible]0.0 |
| Threonine | 3[illegible].7 | 15 0 |
| Serine | 60 0 | 33.5 |
| Glutamic acid | 64 2 | 2[illegible] |
| Proline | 40.3 | 10 0 |
| Glycine | 45 4 | 15.0 |
| Alanine | 41 8 | 17.5 |
| Valine | 40.8 | 14 0 |
| Methionine | 10.0 | [illegible] |
| Isoleucine | 10.9 | 7.[illegible] |
| Leucine | 44 6 | 7.5 |
| Tyrosine | 16.0 | 9 0 |
| Phenylalanine | 19 0 | 10.1 |
| | 570.[illegible] | 12.4 |

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Screening of large numbers of refrigerated sera has clearly shown that spontaneous precipitates which dissolve upon heating at 37 C are not infrequently recognized Although the cryoglobulin concentration is usually rather low and appears as a whitish precipitate at the bottom of the tube much higher amounts may be found in a few cases, usually consisting of

single-type monoclonal cryoglobulins or mixed cryoglobulins with an IgM monoclonal component. Cryoprecipitation occurs in a significant number of sera from patients with infectious diseases associated with autoimmune features [3 9] connective tissue diseases [14 15], and lymphoproliferative disorders [4 6] In addition, a symptom complex including purpura-weakness-arthralgias, which is thought to represent a distinct clinical entity has been frequently observed in association with cryoglobulinemia [4 6, 11] Approximately one-third of such patients develop a severe and rapidly progressive form of renal disease [1 5] Cold precipitates have also been described in a group of patients with acute and chronic liver diseases [7]

The 4 cases reported in this paper were no exception as regards their clinical evaluation. Indeed, patient Bu.E. had membranoproliferative glomerulonephritis with progressive renal failure complicating primary mixed cryoglobulinemia, whereas posthepatitis cirrhosis was diagnosed in patient Bu.T The remaining ? cases (Bu.M and Bu.G ), exhibiting lower amounts of circulating cold-precipitable proteins, had congestive heart failure and mitral stenosis, respectively

In view of the consistent rheumatoid factor-activity of the purified cryoglobulins and their isolated IgM components, the role of such mixed cryoglobulins as antigen-antibody complexes of the IgG anti IgG type can no longer be doubted.

The occurrence of cryoglobulinemia in four sisters may hardly be ascribed to coincidence To our knowledge, relatively few observations of familial cryoglobulinemia have been published so far affecting two brothers [ ] or the proband and two sons [13] Although no clear-cut explanation may be given, it seems likely that genetic and or environmental and or infectious factors may play a role in the pathogenesis of the phenomenon.

While a genetic abnormality may be suggested in family Bu., no definite conclusions can be drawn regarding the mode of inheritance of the defect leading to the protein changes An autosomal recessive trait seems, however to be the most likely explanation.

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# Separation of Erythropoietin Responsive Cells in Fetal Mouse Liver

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*Key Words.* Erythropoietin responsive cells · Cell separation · Fetal mouse liver

*Abstract.* The erythropoietin responsive cells (ERC) in suspension cultures ($ERC_{sc}$) of fetal mouse liver and the cells producing erythroid colonies in 2-day plasma clot cultures (CFU-E) sediment at similar rates. However, the sedimented fractions containing the majority of nucleated cells show minimal sensitivity to erythropoietin (Ep) and these cells sediment at a slower rate than the $ERC_{sc}$. The studies suggest that in short-term suspension cultures of fetal mouse liver Ep acts on morphologically unrecognizable cells to increase the number of hemoglobin-synthesizing cells rather than by increasing the quantity of hemoglobin synthesized per cell. This effect is similar to the principal *in vivo* action of Ep.

## *Introduction*

Erythropoiesis is considered to be under the control of erythropoietin (Ep) acting through morphologically unrecognizable cells in an erythropoietin responsive cell (ERC) compartment. These cells are descended from the 'multipotential stem cell' and precede morphologically recognizable erythropoietic cells [1]. In 1970, McCOOL *et al.* [11] were able to separate, on the basis of cell size, a population of ERC in suspension cultures ($ERC_{sc}$) from the majority of nucleated cells in rat bone marrow. During maturation and division of this population of cells, together with

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initiation of hemoglobin synthesis, Ep sensitivity decreased and the sedimentation velocity changed from 6 6 mm/h to that of the majority of the nucleated cells (2.7 mm/h) and eventually to that of the mature erythrocyte (2.1 mm/h) STEPHENSON and AXELRAD [15] have also been able to separate $ERC_{\mu}$ (sedimentation rate 7 7 mm/h) from spleen colony forming units (5 9 mm/h) of fetal mouse liver

With the introduction of clonal assays for ERC, AXELRAD *et al* [2] ISCOVE and SIEBER [9] and HEATH *et al* [8] using mouse bone marrow cells, were able to separate CFU E and burst forming units-erythroid (BFU E) on the basis of cell size CFU E, defined as the cells producing colonies of up to 32 erythroid cells/colony sediment at a faster rate (5.5 6.5 mm/h) than BFU E (3 7–3 9 mm/h) which produce colonies of greater than 32 erythroid cells/colony These cells can also be separated by their erythropoietin sensitivity by their proliferation rates and by the culture time at which the resultant colonies reach maximum size i.e. CFU E after 2–4 days, BFU-E after 7–10 days. In addition TEPPERMAN *et al.* [18] were able to separate two populations of erythroid colony forming cells in human bone marrow one population produced colonies of $< 32$ erythroid cells, the other colonies of $> 32$ erythroid cells. These populations may be analogous to the CFU E and BFU E of mouse tissues. Both populations of erythroid colony forming cells are morphologically unrecognizable in mouse [4] and human marrow [18] MITCHELL and ADAMSON [14] however were unable to separate CFU E (sedimentation rate 7.56 mm/h) from $ERC_{\mu}$ (7.31 mm/h) in rat bone marrow on the basis of size or kinetic properties.

Suspension cultures of fetal mouse liver cells represent a simple inexpensive and sensitive assay for Ep in clinical material [5 6] In the present study cell separation was undertaken in an attempt not so much to separate CFU E from BFU E, although this has not been previously reported for mouse fetal liver cells, but to ascertain whether one or both of these populations could be separated from the $ERC_{\mu}$ Furthermore, if the $ERC_{\mu}$ could be separated from the majority of heme synthesizing cells in fetal mouse liver the results would imply as morphological studies have suggested [3] that the principal mechanism of action of Ep in fetal mouse liver cell cultures is on immature cells to increase the number of heme-synthesizing cells, rather than on more mature cells to increase the amount of hemoglobin synthesized per cell An Ep action on immature cells appears to be the principal mechanism of action of Ep *in vivo* [1 16]

## Materials and Methods

*Cultures.* Suspension cultures of fetal mouse liver cells were carried out as previously described [5, 6]. Livers from embryos of 13–15 days of gestation were disaggregated by aspiration through Pasteur pipette the cells were diluted to $5 \times 10^5$/1 ml culture in Eagle's Minimal Essential Medium (MEM) + 5% Fetal Calf Serum (FCS) and incubated with or without erythropoietin for 24 h in water-saturated atmosphere of 5% $CO_2$ in air. The end point in this method was the 4-hour incorporation of $^{59}Fe$ into hemoglobin from which hematin was specifically extracted with butan-2-one (methyl ethyl ketone) using our modification [6] of the method originally described by Teale [17].

Assays for CFU-E and BFU-E were carried out using the semimicro plasma clot technique described by Axelrad et al. [2] and McLeod et al. [12]. CFU-E (aggregates of 4–32 erythroid cells) were enumerated after 2 days, and BFU-E (aggregates of > 32 cells) after 8 days, of culture.

*Erythropoietin* of activity 75 U/mg was procured by the Department of Physiology University of the Northeast, Corrientes, Argentina, processed by the Hematology Research Laboratories, Children's Hospital of Los Angeles and distributed by the National Heart, Lung and Blood Institute under Research Grant HE-10880. It was further purified by chromatography on Sephadex G-100 as described by Iscove and Sieber [10].

*Cell separation.* Unit gravity cell sedimentation was performed according to the method documented in detail by Miller and Phillips [13] using solutions of 1 and 2% Bovine Serum Albumin (BSA). Preliminary experiments suggested that detoxification of the BSA enhanced cell recovery and improved cellular morphology There fore BSA detoxified with AG-501 X8 ion exchange resin [19] was used throughout the present studies.

The cell separator used ("Sta-put" Johns Scientific Ltd., Toronto, Ont.) consisted of cylindrical glass sedimentation chamber with an inside diameter of 12 cm and height of 9 cm. A conical section tapering from the full diameter of the chamber to that of an 18G needle and with volume of 80 ml was attached to the base of the chamber. Cells were loaded and fractions collected through the 18G needle at the base of the chamber A stainless steel baffle was positioned at the bottom of the cone to prevent disturbances during loading and emptying.

To load the cell separator, 50 ml of phosphate-buffered saline, pH 7.3, was run into the chamber from the base followed by 20 ml of 0.5% BSA in which total of approximately $1 \times 10^8$ cells were suspended. Subsequently 20 ml of 0.3% BSA and 700 ml of 1–2% BSA in phosphate-buffered saline gradient were added. After 4 h sedimentation at 4 °C, the cone volume was discarded and 15-ml fractions (corresponding to chamber depth of 1.3 mm) were run out through the base of the chamber at 2 ml/min. After centrifugation the cells in each fraction were washed twice with Eagle MEM + 5% FCS and the number of cells determined by use of Coulter Model S electronic cell counter After further centrifugation, small part of the cell pellet was smeared onto a microscope slide. Smears from alternate fractions were stained, for cytological analysis, with Jenner-Giemsa or benzidine count erstained with hematoxylin [12]. The remaining cells from each fraction were resus-

initiation of hemoglobin synthesis Ep sensitivity decreased and the sedimentation velocity changed from 6 6 mm/h to that of the majority of the nucleated cells (2.7 mm/h) and eventually to that of the mature erythrocyte (2 1 mm/h) STEPHENSON and AXELRAD [15] have also been able to separate $ERC_{ss}$ (sedimentation rate 7 7 mm/h) from spleen colony forming units (5 9 mm/h) of fetal mouse liver

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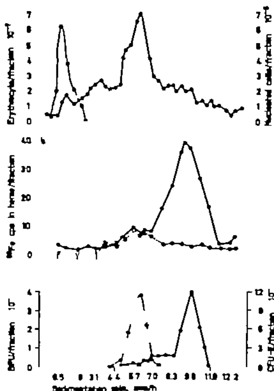


*Fig 1* Unit gravity sedimentation of fetal mouse liver cells at 4 °C for 4 h. Total nucleated cells (●) and erythrocytes (O). *b* Incorporation of $^{59}$Fe into hemoglobin in the absence (O) or presence (●) of 200 mU/culture of Ep CFU-E (●) and BFU-E (O) in the presence of 200 mU/culture of Ep. Mean results of three experiments.

## *Discussion*

Cells responsive to Ep in suspension cultures (sedimentation rate 8 67 mm/h) were clearly separable from the majority of nucleated cells (sedimentation rate 4 67 mm/h) in fetal mouse liver In the absence of Ep, the major peak of nucleated cells (fig. 1a) showed significant $^{59}$Fe incorporation which was not enhanced when Ep was present in the cultures (fig. 1b). CFU-E sedimented at a rate (8.67 mm/h) comparable to ERC$_{14}$ and considerably faster than BFU-E (6.1 mm/h) (fig. 1b, c).

pended in appropriate volumes of Eagle's MEM + 5% FCS and then cultured, in duplicate, as described above. By establishing an Ep dose-response relationship on the original cell suspension and on a suspension prepared from the pooled fractions no evidence was obtained that the Ep sensitivity of the cells declined during the necessary 4–5 h of sedimentation prior to culturing [see also reference 7].

Each fraction was assayed for suspension culture Ep-sensitive cells, CFU-E and BFU-E. Where possible, two different cell concentrations (between 2–10 × $10^5$ cells/suspension culture and 4–40 × $10^4$ cells/0.1 ml plasma clot culture) were incubated with 0 or two or three concentrations of Ep. For a given Ep concentration, the number of colonies/$10^5$ cells was independent of cell concentration over the range tested. Data in figure 1 are expressed per fraction calculated from the total number of cells in each fraction and the proportion of each fraction used per culture. The results from Ep-stimulated cultures were obtained from cultures containing 200 mU Ep/culture – a concentration which produced maximal stimulation of $ERC_{Fe}$ and produced approximately 100 CFU-E and 10 BFU-E per $10^5$ unfractionated fetal liver cells.

## *Results*

The mean results of three unit gravity cell sedimentation experiments are shown in figure 1 and of seven further experiments in table I. Approximately 60–65% of the initial number of cells were recovered in each experiment.

The majority of nucleated cells (consisting of 70–80% basophilic, polychromatic and orthochromatic normoblasts) were found in a broad peak with a modal sedimentation rate of between 3.47 and 6.7 mm/h (fig. 1a, table I). In cultures without Ep a broad peak of $^{59}Fe$ incorporation 7–8 times background with a modal sedimentation rate of 6.36 mm/h was observed. This radioiron incorporation was not enhanced by the presence of Ep in the cultures (fig. 1b). Cells responding to Ep in suspension cultures by an increase in $^{59}Fe$ incorporation into hemoglobin showed a broad distribution with a modal sedimentation rate of 8.42–9.4 mm/h (fig. 1b, table I). This peak consisted of up to 60% benzidine-negative cells.

CFU-E sedimented at a modal rate of 8.42–9.6 mm/h and BFU-E at 6.1 mm/h (fig. 1c). No colonies were seen in the absence of Ep. The sedimentation profile was not influenced by fetal liver cellularity over the range 8.4–26.0 × $10^6$ cells/liver corresponding to a fetal age of 13–15 days (table I).

Few hepatocytes were seen in any of the fractions (and were seldom observed in unfractionated suspensions) suggesting that these cells did not survive the disaggregation procedure.

Suspension cultures of hematopoietic cells have been widely used to study erythropoiesis. Nevertheless, there is little information regarding the properties of the ERC in these systems and how they relate to the CFU-E and BFU-E assayed in the more recently introduced clonal techniques. The present studies suggest that CFU-E and $ERC_{ss}$ are probably of the same cell population. Although the possibility cannot be completely excluded that they represent different populations of similar size, such a possibility seems unlikely MITCHELL and ADAMSON [14] for example, have shown that the two populations show similar sensitivity to tritiated thymidine and our preliminary data would suggest they also cannot be separated on the basis of sensitivity to hydroxyurea.

To a certain extent it is perhaps not surprising that we have been unable to separate CFU-E and the suspension culture Ep-sensitive cells as the incubation periods in both techniques were short term 24 h for suspension culture, 48 h for CFU-E. It will be of interest to determine if long-term suspension cultures (7–10 days) reveal the presence of an Ep-sensitive cell comparable with the BFU-E. Preliminary studies have suggested a second 'wave of heme synthesis in suspension cultures maintained for 7 days.

The cell separation studies reported above and detailed morphological investigations [3] show that in short term suspension cultures of fetal mouse liver cells, Ep acts to enhance cell division in morphologically unrecognizable precursor cells (possibly CFU-E) leading to an increase in the number of cells synthesizing hemoglobin. These observations suggest, therefore, that the mode of action of Ep in fetal mouse liver cells *in vitro* is similar to the principal mechanism of action of the hormone *in vivo* [1 16]

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*Table 1* Sedimentation rates of $ERC_{50}$, CFU-E and BFU-E of fetal mouse liver cells

| Mean nucleated cell count $\times 10^6$/liver | Sedimentation rate, mm/h Nucleated cells | $ERC_{50}$ | Endogenous $^{59}Fe$ incorporation | CFU-E | BFU-E |
|---|---|---|---|---|---|
| 22.2 | 5.45 | 8.91 | 6.93 | ND | ND |
| 23.7 | 3.47 | 8.42 | 5.44 | ND | ND |
| 8.4 | 3.96 | ND | ND | 8.91 | 6.1 |
| 12.9 | 3.96 | 8.42 | 6.44 | 8.42 | 6.1 |
| 10.8 | 5.45 | 8.42 | 6.44 | ND | ND |
| 16.0 | 4.95 | 8.91 | 6.44 | ND | ND |
| 9.6 | 5.45 | 8.91 | 6.44 | ND | ND |
| Mean (± SE) | 4.67 ± 0.32 | 8.67 ± 0.11 | 6.36 ± 0.2 | 8.67 ± 0.81 | 6.1 |

ND = Not determined.

These results, therefore, in general confirm the studies of other workers [2, 8, 9, 11, 15, 18] that several distinct Ep-sensitive cell populations can be separated from suspensions of various hemopoietic tissues. The differences in the absolute sedimentation rates may reflect tissue-specific cell sizes or minor differences in technique.

WORTON *et al.* [19] have pointed out that extreme caution is required when interpreting the results of cell separation studies. Each fraction is probably altered not only in the relative concentration of responding cells (e.g., CFU-E) but also in the number of 'helper' or 'inhibitor' cells. Thus the number of colonies obtained from each fraction may be dependent on at least three parameters. Although no specific 'helper' or 'inhibitor' cells have yet been identified for CFU-E or BFU-E [8], HEATH *et al.* [8] demonstrated that the number of BFU-E obtained from fractionated mouse bone marrow was not always proportional to the cell number plated. This particular aspect did not represent a major area of study in the present investigations using fetal liver cells, but no evidence for this effect was observed over the somewhat limited range of cell concentrations investigated. Furthermore, detailed investigations [8] do confirm that unit gravity separation of CFU-E and BFU-E, similar to that now reported, is indicative of real differences in the size of these two cells and is not due to artifacts introduced by the cell separation procedure.

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# Different Composition of the Erythropoietic Tissue in Bone Marrow, Spleen and Liver in Myelofibrosis

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*Key Words.* Bone marrow Extramedullary erythropoiesis Liver Myelofibrosis Spleen

*Abstract.* Aspirates from bone marrow spleen and liver were analysed in 10 untreated patients with idiopathic myelofibrosis (MF). The proportion of erythroblasts was higher in the spleen and the liver than in the bone marrow The mitotic indices of the erythropoietic precursor cells were subnormal in the extramedullary sites and significantly lower in the liver compared with the spleen. There was 'shift to the left' within the liver erythropoiesis and significant megaloblastosis in the spleen. The same tendencies have formerly been found in patients with chronic myeloid leukemia and it is suggested that the discrepancies may be due to differences in the microenvironment of the erythropoietic cells.

Enlargement of the spleen and some degree of hepatomegaly are regularly present in MF Although the extramedullary erythroblasts give a considerable contribution to the erythropoietic pool the successive development of anemia is a characteristic clinical feature of MF There is little information of the function and the composition of the erythropoietic tissue in the liver in myeloproliferative disorders and the present work was undertaken to investigate whether or not the morphology and the mitotic activity of erythroblasts of the spleen and the liver might indicate some abnormalities in the extramedullary production of red cells in MF Comparisons with earlier investigations of CML patients [10–13] may also give some information on the influence of the microenvironment on the erythroblasts.

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*Table I* Composition of the erythropoietic pools in the bone marrow spleen and liver of 10 MF patients, 15 CML patients and in the bone marrow of 17 healthy controls (figures within parentheses are based on only 5 patients)

| | Bone marrow | Spleen | Liver |
|---|---|---|---|
| | $Q_1$ $Q_2$ $Q_3$ | $Q_1$ $Q_2$ $Q_3$ | $Q_1$ $Q_2$ $Q_3$ |
| *Erythroblasts* (% of precursor cells) | | | |
| MF | 16.7–35.9–64.3 | 49.5–64.8–71 6 | 52.8–79.3–88 0 |
| CML | 3.1–6.3–11.6 | 8.6–11.9–17.9 | 6.0–13.6–17 4 |
| Normals | 38.4–42.4–47.5 | | |
| *Mitoses* (% of all erythroblasts) | | | |
| MF | (1.8–2.6–3.1) | 1.8–[illegible].3–3 5 | 1.5–1.8–2.7 |
| CML | 3 2–3.5–4 1 | 2.1–2.5–3.0 | 2.3–[illegible].6–2 9 |
| Normals | 2.8–2.9–3.3 | | |
| *Basophilic erythroblasts* (% of all erythroblasts) | | | |
| MF | (24 4–31.6–48.4) | 31.5–37 7–45.7 | 37.9–45.3–50.6 |
| CML | 32.6–37.8–45.0 | 30.6–38.4–40 8 | 32.9–40.0–47.2 |
| Normals | 29 5–30.5–31.9 | | |
| *Megaloblasts* (% of all erythroblasts) | | | |
| MF | (3.8–4.8–23 4) | 7.0–9.9–19 7 | 3.7–5.2–8.3 |
| CML | 1.2–2.2 3.6 | [illegible].9–5.3–8 6 | 2 2 3.8–5.2 |
| Normals | 1.2 1 7–2.3 | | |

marrow the spleen and the liver of the patients compared with the bone marrow of the normals ($p < 0.01$). There were more megaloblasts in the spleen than in the liver of the patients ($p < 0.02$ table I).

## *Discussion*

Myelofibrosis (MF) is characterized by an inadequacy of the bone marrow concomitant with enlargement of the liver and the spleen associated with extramedullary hemopoiesis. A considerable amount of extramedullary erythropoietic tissue may not prevent the successive development of anemia, however Many different factors seem to contribute to this usually refractory anemia. Thus, hemodilution and spleen sequestration are prominent factors especially in cases of MF with pronounced

## Material and Methods

*Patients.* 10 untreated patients with a recent diagnosis of MF were examined. There were 5 men and 5 women with a median age of 62 (range 44–82). Some pertinent hematological data were B-Hb 6.1–13.7 g/dl, Ery-MCV 87–100 fl, WBC 9–14 × $10^9$/l, platelets 63–672 × $10^9$/l, B-histamine 0.4–7.2 µmol/l.

*Controls.* (I) 15 untreated patients with a recent diagnosis of CML. Detailed descriptions have been given elsewhere [11–13]. (II) 7 male and 10 female probands without perceivable hematologic disorders and with clinical diagnoses such as spondylolisthesis, cervical disc prolapse, adipositas, psychiatric disorders and unverified hypogonadismus served as normals. Their median age was 50 years. B-Hb 12.1–16.6 g/dl and WBC 2.2–8.3 × $10^9$/l.

*Morphological investigations.* Bone marrow was obtained through sternal puncture and material from the liver and the spleen through fine-needle biopsy as described by Söderström [15]. Aspirates from the three organs were obtained on the same occasion and the smears were stained with May-Grünwald-Giemsa. In order to reduce the influence of pronounced admixture of peripheral blood the proportions of erythroblasts were determined by examination of 1,000 precursor cells, i.e., myeloblasts, myelocytes, erythroblasts and megakaryocytes. Neither cells belonging to the liver parenchyma, the splenic lymphocytes and pulp cells nor mature leukocytes were thus included in these calculations. In all smears, except 5 bone marrow smears which were too hypocellular to permit separate studies of the erythropoiesis, 1,000 erythroblasts were examined and divided into normoblasts and megaloblasts according to criteria given by Heilmeyer and Begemann [4]. Procrythroblasts and basophilic erythroblasts were then pooled into one group and denominated basophilic erythroblasts. All differential counts were made by the author. It proved impossible to carry out a blind analysis of the smears from the bone marrow, the spleen and the liver, since the splenic pulp cells and the liver parenchyma cells revealed the origin of the smears.

*Statistics.* The results are expressed as median ($Q_2$) and interquartile range ($Q_1$–$Q_3$). The Wilcoxon matched-pairs signed-ranks test and the Mann-Whitney U test were used to assert significance of the results.

## Results

There were significantly more erythroblasts among the precursor cells in the spleen ($p < 0.05$) and the liver ($p < 0.02$) compared with the bone marrow of the MF patients and also with the bone marrow of the normals ($p < 0.01$, table I). The mitotic activity was subnormal in the liver of the patients ($p < 0.01$, table I). The proportion of basophilic erythroblasts was significantly higher in the liver than in the spleen of the patients ($p < 0.05$, table I).

The percentage of megaloblasts was significantly higher in the bone

forms of myeloproliferative disorders [10–13 15 16] The development of megaloblasts thus confirms the other morphologic signs of an ineffective erythropoiesis.

Because of the great variability of the histologic pattern in the bone marrow of the MF patients the conclusions from marrow aspirates ought to be prudential [6, 8, 18] The extramedullary erythropoiesis shows morphologic abnormalities indicating an ineffective production of erythroblasts. There are also certain differences between the composition of the spleen and the liver erythropoiesis. The same tendencies, although not so pronounced, have formerly been found in CML and the discrepancies described in this investigation may well be explained by environmental differences. A seeding of more or less defective precursor cells from the bone marrow into the liver and the spleen, as proposed by others [6] cannot be ruled out, however

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splenomegaly [1–3] A rather high incidence of megaloblastic anemia due to folate deficiency among the MF patients has also been reported [5] Studies of red cell kinetics have shown that the splenic erythropoiesis in MF is considerably insufficient [3 9 14 18] and often combined with a striking hemolysis. Pure morphologic investigations of the spleen erythropoiesis have also given evidence of an ineffective production of erythroblasts in MF [15 16] and in other myeloproliferative disorders such as CML [10–13] and polythemia vera [15 16]

It has previously been demonstrated that the proportion of erythroblasts within the hemopoietic tissue is larger in the spleen than in the bone marrow of the MF patients [8 16 18] and this is verified in this investigation There are also more erythroblasts among the precursor cells in the liver and the same results have been found in CML patients [10–13] The higher proportion of erythroblasts in the liver and the spleen could be due to sampling errors, because erythroblasts may be more readily aspirated [18] and/or the true prevalence of myelopoiesis may be underrated due to rupture of the myelocytes [16] There is some experimental evidence that the splenic stroma may favor the differentiation of the erythroid cells [7 17 19] however and that the discrepancies observed are caused by environmental differences

Previous investigations of CML patients [10 12, 13] have shown low mitotic indices of the erythroblasts in the extramedullary hemopoietic tissue and the differences were more pronounced in the early phase of the disease In MF the hemopoietic tissue in the liver and the spleen is not dominating and the function of the organs remains normal for a long time [8 16 18] In the present investigation the mitotic indices of the liver erythroblasts were found to be very low A pronounced immune response and/or an unfavorable microenvironment may possibly explain the low indices observed [10 13]

A raised frequency of early erythroid precursors, i.e., basophilic erythroblasts was found especially in the liver aspirates. There could be a maturation arrest of the erythroblasts but it is also possible that there is a destruction of the later stages as in ineffective hemopoiesis.

Although megaloblastic anemia due to folate deficiency is not uncommon [5] this was not the case in the patients of this investigation Megaloblastic changes, indicating a disturbed DNA synthesis, were constant findings in all the smears, however

The highest frequencies were found in the spleen which is in agreement with previous observations in aspirates from patients with various

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# Benign Sickle Cell Anemia in Israeli-Arabs with High Red Cell 2,3 Diphosphoglycerate

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*Key Words.* Sickle cell anemia · Hemoglobin S · 2,3 Diphosphoglycerate

*Abstract.* Arabs living near the Sea of Galilee were found to be homozygous for hemoglobin S. Studies of solubility, mechanical precipitability, electrophoretic mobility on starch-gel and citrate agar media, minimum gelling concentration, and peptide mapping of the hemoglobin β-chain confirmed complete identity of the hemoglobin with that found in Afro-American hemoglobin S homozygotes. A comparison of Arab Hb S homozygotes with Afro-American Hb S patients showed no significant differences in hemoglobin levels, red cell indices or morphology. Hb F averaged 4.4% in Arab patients. The 2,3 diphosphoglycerate levels were increased approximately twofold in Arabs, whereas in Afro-Americans, it was increased by only 7% in females and 20% in males.

## *Introduction*

The origins of sickle cell anemia have been investigated by both historical and medical-anthropological methods. A recent synthesis of these studies [1] concludes that the hemoglobin S gene probably appeared as an isolated mutation in Equatorial Africa, and spread to parts of Southern Europe and the Arabian peninsula as a result of population movements, the spread of falciparum malaria, and interactions with certain other genes. It is likely that the inhabitants of the Arabian peninsula played an important role in the dissemination of sickle cell syndromes, and that

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*Table 1* Hematologic values for Galilee Arabs and Afro-Americans with sickle cell anemia and sickle trait (mean ± SD)

| | Hb, g/dl | Hb F % | MCV fl | MCHC, % | ATP μmol/g Hb | S in S-trait | Reticulocytes, % |
|---|---|---|---|---|---|---|---|
| Arab controls | | | | | | | |
| Male (n = 5) | 15.46 ±1.15 | | | | | | |
| | | 1.67 ±0.56 | 86.2 ±4.4 | 33.38 ±0.87 | 3.37 ±0.45 | – | – |
| Female (n = 5) | 12.84 ±0.44 | | | | | | |
| Arab Hb SS | | | | | | | |
| Male (n = 3) | 8.90 ± 2 | | | | | | |
| | | 4.40 ±2.87 | 84.6 ±8.2 | 33.43 ±1.55 | 4.13 ±0.73 | – | 8.7 ±5.1 |
| Female (n = 5) | 8.7 ±0.9 | | | | | | |
| Arab S-trait | | | | | | | |
| Male (n = 4) | 14.77 ±1.63 | 1.43 ±0.48 | 85.0 ±5.6 | 33.35 ±0.56 | 3.22 ±0.26 | 41.0 ±3.3 | – |
| Afro-American controls | | | | | | | |
| Male (n = 11) | 15.42 ±1.13 | – | 87 ±5 | 34 ±2 | 3.31 ±0.40 | | – |
| Female (n = 8) | 13.22 ±1.17 | | – | – | – | | |
| Afro-American Hb SS | | | | | | | |
| Male (n = 13) | 8.54 ±1.81 | 5.8 ±4.9 | | | | | |
| | | | 91.0 ±8.0 | 33.33 ±2.55 | 4.48 ±0.70 | | 9.2 ±3.8 |
| Female (n = 11) | 7.63 ±0.9 | 6.0 ±4.2 | | | | | |

* Normal values from Coulter Counter Co.

maps of isolated β-chains were made by established methods [11, 12], and peptides were analyzed on a Jeolco Model JLC-6AH amino acid analyzer. A limited physical examination on each subject was performed, as was review of hospital records over 16-year period and interviews with the subjects. These Arab patients were compared with Afro-Americans with sickle cell anemia living in New York City as

slave trading brought hemoglobin S to the Arabian peninsula and then as far east as India, and by conquest, to Southern Europe.

It has been observed that in non-African populations with sickle cell anemia, the disease has a milder course than the same illness occurring in persons living on the African Continent or in America [2, 3]. This finding has often been attributed to relatively high levels of fetal hemoglobin. However, the variation in fetal hemoglobin levels does not explain the extreme variability of symptoms found in many individuals who possess the same chemical abnormality of hemoglobin structure and very similar levels of HB F. This enigma applies both to patients within the same ethnic group and when comparisons are made between geographically distinct groups. In short, the pathophysiology of benign sickle cell anemia remains unknown.

This study is concerned with a non-African population with sickle cell disease and sickle cell trait living in Northern Israel and who were first reported in 1955 [4]. In 1974, this population was restudied for the correlation of hemoglobin S with blood group phenotypes considered to be specific for African ancestry [5]. The disease was classified as benign because of the relatively rare occurrence of vasculo-occlusive-infarctive crises with pain, hemolytic crises, sequestration syndromes or hypoplastic crises.

The present study was designed to extend the original observations by physicochemical analysis of the hemoglobin S present in this population, to further the clinical studies and, in particular, to evaluate possible factors to account for the benign expression of sickle cell disease in this population.

## *Methods*

Heparinized and clotted whole blood specimens were obtained from inhabitants of the village of Wadi Hamam near the Sea of Galilee. Aliquots for 2,3 diphosphoglycerate (2,3 DPG) and red cell ATP were immediately precipitated in cold trichlor acetic acid. Determinations of 2,3 DPG and ATP were made within 24 h using Sigma Kits No. 366 UV and 35-UV (Sigma, St. Louis, Mo.).

Aliquots of the samples were used for routine hematological studies on a Model S Coulter Counter, reticulocyte counts and biochemical profiles (SMA 6).

Lysates were made according to the method of Drabkin [6] and starch-gel [7] and citrate agar [8] electrophoresis were performed at pH 8.6 and 6.5, respectively. Minimum gelling concentrations (MGC) and mechanical stability studies of oxyhemoglobin were performed as described previously [9, 10]. Solubility studies of whole blood were performed using the Ortho Laboratories Sickledex Kit. Peptide

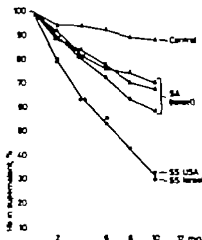



*Fig 1* Mechanical precipitation rates of Hb S from Galilee Arabs and from Afro-Americans. Control sample is a lysate of normal blood, Hb S-A heterozygotes are Israeli Arab samples with percent Hb S from 36 to 4?% Samples were agitated as described previously [10] in 0 15 M KPO buffer pH 7.35 at 24–26 °C.

significant at the 0 1 /alevel (t = 6 4 d.f. = 11), and for females, significance is at the 7% level (t = 2.86 d.f = 13). Within the Afro-American group the mean 2,3 DPG level in males with sickle cell anemia was significantly higher than in the control group ($p < 0.01$ t = 3 1 d.f. = 18) However for females, the difference was not significant (t = 1 1 d.f. = 18). These data indicate that the Arabs from the Wadi Hamam village have approximately a twofold increase in red cell 2,3 DPG levels, whereas the sickle cell patients from New York City had an increase of about 20% in males and, perhaps, none in females.

### *Discussion*

Inspection of the affected inhabitants of the village of Wadi Hamam revealed only slight pallor and very minimal scleral icterus. There was no evidence of healed or active leg ulceration. None of the patients offered any history of frequent episodes of pains or need to seek frequent medical attention. A review of hospitalizations since 1960 revealed occasional hospitalizations for typical syndromes associated with sickle cell anemia.

*Table II* Red cell 2,3 DPG levels in Galilee Arabs and American Blacks normals and Hb SS patients

| | Age (mean ± SD) | n | 2,3 DPG µmol/g Hb |
|---|---|---|---|
| Normal Arabs | | | |
| Males | [illegible]2.5 ± 9.5 | 5 | 13.84 ± 0.59 |
| Females | | 5 | 14 62 ± 1.38 |
| Arab Hb SS patients | | | |
| Males | 16.7 ± 6.8 | 3 | 30.33 ± 3.05 |
| Females | | 5 | [illegible]5.4 ± 7.38 |
| Black Hb SS patients | | | |
| Males | 3[illegible]4 ± 9 1 | 12 | 18.[illegible]3 ± [illegible].47 |
| Females | | 10 | 17.51 ± 2.4[illegible] |
| Normal Blacks | | | |
| Males | [illegible]8 7 ± 10 | 8 | 15.09 ± 1.37 |
| Females | | 10 | 16.3 ± 1.83 |

seen in the Heredity clinic of the Albert Einstein College of Medicine. Comparisons between various groups were tested for significance using the Student's t test with Bessel's correction for small samples [13]

## *Results*

The MGC of hemoglobin S lysates from the Galilee Arabs was identical to the MGC of lysates from Afro-Americans 24 8 ± 1.2 vs. 23 7 ± 0 7 g%. Complete identity between the Arab and Afro-American samples was also seen in the following studies electrophoresis on starch gel and citrate agar solubility mechanical precipitation (fig. 1) peptide mapping and amino acid analysis, Hb F levels and the routine hematologic studies (table I) The peripheral smear of the Arab homozygotes revealed many irreversibly sickled cells, polychromatophilia and Howell Jolly bodies and resembled smears from American patients. The reticulocyte counts in the Arab patients were not significantly different from those of Afro-Americans. 9.2% ± 3.8 vs. 8 7 ± 5 1%.

The 2,3 DPG levels for the control groups do not differ between Israeli Arabs and Afro-Americans (table II) Significant differences were noted between the two groups of sickle cell patients. For males, the difference is

spect, as well, the Galilee Arabs differ from the Shiite Arabs in whom Perrine found a 2,3 DPG elevation of about 25% over the control values. Our data for 2,3 DPG red cell levels in Afro-Americans are quite similar to other recent reports [15–17]. The Wadi Hamam village is located below sea level, and the controls had normal levels of 2,3 DPG. In addition, blood chemistries disclosed no abnormalities of renal function or calcium-phosphate metabolism.

The allosteric effector 2,3 DPG has been shown to lower the minimum gelling concentration of Hb S which indicates that it favors the polymerization reaction [18–20]. This finding was initially interpreted as proof of a conformational effect mediated by the binding of this organic phosphate [20] in addition to its well known stabilization of the T state [21]. More recent data seem to indicate that 2,3 DPG might favor polymerization exclusively by a pH effect [22]. Moreover, high levels of 2,3 DPG have been shown to increase the red cell MCHC and lower intracellular pH [23]. All these effects would be likely to enhance sickling and contribute to the frequency or severity of crises. However, the overall effects of 2,3 DPG on sickling of red cells *in vivo* is still a matter for further investigation, and no definitive description of the effects of 2,3 DPG is at hand.

The results of this study support the previous conclusion of RACHMILEWITZ *et al.* [5] that this group of Arabs derived their gene for Hb S from African sources. The identity of the Arab and Afro-American Hb S in all physicochemical studies support this contention. The presence of African blood group markers in these patients, the finding of Hb C in a nearby village of northern Israeli Arabs [24] and the present studies, all indicate with reasonable certainty that an admixture of African genes has occurred in these Levantine populations.

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These episodes ranged between 0 and 4 per patient over a 14-year period. No patient required chronic transfusion there was no gross evidence of ocular or neurological damage one female patient delivered three children without complications other than a bout of pyelonephritis during one pregnancy Thus the label of relatively benign sickle cell disease seems to be appropriate to this group of patients.

It is of interest that although the Hb F levels in these Arab patients are similar to Afro-American patients in New York City the levels of fetal hemoglobin differ markedly from previous studies in Arabs with sickle cell anemia. PERRINE *et al* [3] reported a mean Hb F level of 18 9% in Saudi Shiite Arabs, and GELPI [2] who studied the same population, obtained a mean value of 10% with a range of 1 4–24 7%. The low levels of fetal hemoglobin exclude this as an ameliorating factor in the disease of these Galilee Arabs These patients, therefore, stand as a distinct group from the Shiite Arabs of the Saudi Peninsula

The present studies strongly suggest the identity of Arab and Afro-American hemoglobin S on the basis of several physical properties as well as their chemical analysis Hemoglobin S from both groups of patients has the same electrophoretic mobility at two different pH values and on two different support media as well as the same solubility minimum gelling concentration and oxyhemoglobin precipitation rates. Thus, it is not surprising that peptide mapping and amino acid analysis should also yield identical results However these studies do not necessarily exclude the presence of an electrophoretically silent substitution elsewhere in the $\beta$-chain. In addition a silent mutation might also have occurred in the $\alpha$ chain. Indeed, mutations at two $\beta$-chain loci have been reported [14] but these are exceedingly rare In the instance of Hb $C_{Harlem}$ ($\beta$6Glu→Val and $\beta$73Asp→Asn) both the gelling and mechanical precipitation properties have been altered as a result of the second mutation [10 14] In the remote case that a silent substitution exists in the hemoglobin S found among the Arab patients, the mutation does not modify any of the abnormal or normal erythrocytic properties that we have examined.

Identity between Afro-American and Arab Hb S homozygotes is also observed in routine hematological studies in which total hemoglobin levels, red cell indices, red cell ATP and Hb F levels do not differ significantly

At present, the high red cell 2,3 DPG values found in these patients is the principal distinguishing feature elucidated by these studies In this re-

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# Short Communications

Acta haemat. 59: 246–249 (1978)

# Waldenström's Macroglobulinaemia Presented as Pleurisy of Unknown Origin

## A Case Report

ANTHONY SIMOS, P. ASSEO, G. HADJIDIMITRIOU, P. GARZONIS and D. COLOVOS

Medical Unit of the Chest Hospital of Athens, Athens

*Key Words.* Waldenström's Macroglobulinaemia · Pleural effusion · Ascites · IgM · Immunoelectrophoresis · Bone marrow aspiration · Bone biopsy · Pleural biopsy · Peripheral neuropathy · Protein electrophoresis

*Abstract.* This report describes an unusual case of Waldenström's macroglobulinaemia, in which the sole demonstrable clinical manifestations were pleural effusion and ascites. Bone marrow aspirates and bone biopsy revealed no increase in lymphocytes. The case responded to a combination therapy of melphalan, cyclophosphamide and prednisolone.

## *Introduction*

Since 1944 when the first description of the condition of Waldenström's macroglobulinaemia set forth, a number of reports with special emphasis in chest findings have been published.

In the present report we describe the clinical course of a patient with Waldenström's macroglobulinaemia, whose sole manifestation of the disease was related to pulmonary involvement without any demonstrable bone marrow infiltration or involvement.

## *Case Report*

M. A., a 67-year-old male, previously in good health, noticed for the first time in November 1974 weakness and dyspnoea of progressive character. In January

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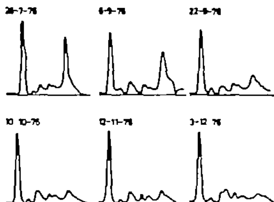



*Fig 1* Densitometric scans of serum-protein electrophoresis.

1975, ascites developed and the patient was admitted to General Hospital. According to enquiries, liver function tests, liver biopsy bone marrow aspirates and liver scan, were negative. Symptomatic treatment was given and the patient was discharged. 1 August 1975 the patient presented productive cough, anorexia, progressive dyspnoea and pain located on the base of the left hemithorax H was re-admitted to the hospital. Chest X-rays revealed free fluid in the pleural cavity which was aspirated and the patient was discharged again without any specific treatment. Ever since, the patient has had multiple admissions because of dyspnoea, and each time the reproduced fluid was aspirated as before.

In July 1976, the patient was admitted into our medical unit because of similar episode. On examination he appeared weak and pale. The following findings and observations were made: fundoscopic examination was normal· the abdomen was soft and the liver and spleen were not palpable; no glands were found.

Examination of the chest revealed decreased breath sounds, dullness over the left lower hemithorax. Chest X-ray demonstrated the presence of pleural effusion. The heart was normal. The neurological examination revealed findings compatible with peripheral neuropathy No haemorrhagic erythema was present. Repeated sputum examination for acid-fast bacilli, fungi and malignant cells were negative. The bone marrow aspiration and bone biopsy were normal without evidence of elevated numbers of either lymphocytes or plasma cells. N Bence-Jones protein was found. Further X-ray films revealed no bony lesions, pleural biopsy revealed lymphocytoid infiltration. The pleural fluid was exudate with 100% lymphocytes No abnormal cells were seen (Papanicolaou tests). The bronchoscopy showed nothing special. The serum electrophoresis demonstrated an abnormal peak in the gamma region. Serum total proteins were 8.5 g% of which 4.9 were γ-globulin. Immunoelectrophoresis showed an abnormal arc with anti-IgM serum Immunoglobulin determination showed IgM = 4 400 mg% (normal levels 50–267 mg%). The SIA test was strongly positive. Blood count, liver function tests, serum transaminases, blood urea

# Short Communications

Acta haemat. 59 46-249 (1978)

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nitrogen, blood sugar calcium and phosphorus, BSP retention in 45' were within the normal limits. The ESR was high > 100 mm. The diagnosis of Waldenström's macroglobulinaemia was made. Melphalan, cyclophosphamide and prednisolone were given. The combination therapy for 4 days was repeated every 3 weeks and there was progressive clinical improvement. The pleural fluid and dyspnoea disappeared, the peak of γ-globulin diminished progressively (fig. 1)

## *Discussion*

The diagnosis of Waldenström's macroglobulinaemia was based in this case on the physicochemical and immunological properties of the abnormal protein.

Pleural and pulmonary manifestations of Waldenström's disease are unusual and very rare. The first report of pulmonary involvement in Waldenström's disease was by Noach [1956]. Since then, the occurrence of pulmonary infiltration during the course of the disease has been mentioned by others [Furgerson *et al.*, 1963; Strunge 1969; Bollinelli *et al.* 1970; Neiman *et al.* 1973]. Chest findings may be subdivided as follows: Infection manifestations, including bronchitis, pneumonias, etc. Parenchymal involvement. Bronchial infiltration producing atelectasis. Pleural effusion. This may be unilateral or bilateral and may coexist with ascites or pericardial effusions.

In our case, several of the above mentioned pulmonary findings were observed. It is worth noticing that, when the ascites appeared, the chest X ray was normal and the liver function tests and the liver biopsy were within normal limits.

The neurological examination revealed peripheral neuropathy which is supposed to be one of the most common neurological findings. It is of interest that the patient had no bone marrow involvement and pulmonary infiltration was the sole manifestation of the disease. The only area in which lymphocytic infiltration could be found was in the pleural specimen obtained by biopsy and in the pleural fluid.

Using melphalan in combination with cyclophosphamide and steroids, we obtained progressive symptomatic improvement and a reduction in the amount of circulating macroglobulin. The main feature that we wish to stress is the response to a combination of melphalan, cyclophosphamide and prednisolone. This improvement, however, indicates clearly that the above therapy reduced the number of the abnormal cells. On the contrary, the symptoms of peripheral neuropathy were aggravated.

*Table I.* Results of skin tests

| Material injected intradermally | Reaction |
|---|---|
| Autologous whole blood | + |
| Homologous whole blood | + |
| Autologous saline suspension of washed red cells | + |
| Homologous saline suspension of washed red cells | + |
| Autologous hemoglobin | + |
| Homologous hemoglobin | + |
| Autologous red cell stroma | – |
| Homologous red cell stroma | – |
| Autologous serum | – |
| Autologous plasma | – |
| Autologous saline suspension of lyzed platelets | – |
| Autologous saline suspension of lyzed leukocytes | – |
| PPD | – |
| Histamine | |
| Isotonic saline | |

sodes of spontaneous painful ecchymoses on his extremities since 2 years. The ecchymoses were preceded by localized pain, burning, itching and sensation at the affected site. He has had epistaxis on two occasions and melena and syncopal attack once during the appearance of painful ecchymoses. He also suffered from sexual impotence since 2 years.

There was no pathological finding on physical examination on admission. On psychiatric examination, a strong neurotic personality was established.

His hematological findings, including bleeding time (Ivy), platelet count, platelet aggregation and coagulation tests were within normal limits.

After the intradermal injection of autologous and homologous whole blood, saline suspension of washed red cells and hemoglobin solution, a painful edematous red plaque developed at the site of injection within 1 h. The red plaque turned into ecchymoses in 24 h (fig. 1). The skin reaction induced with hemoglobin solution was much larger than that induced with whole blood and washed red cells.

After the injection of the same amount of autologous and homologous red cell stroma, autologous plasma, serum, saline suspension of lyzed leukocytes, saline suspension of lyzed platelets and isotonic saline no reaction occurred (table I).

## Discussion

The most interesting features in this patient were being a male and having autoerythrocyte sensitization due to hemoglobin. This syndrome has until now only occurred in women [1–3]. To date, we were not able

Acta haemat. 59 250-253 (1978)

# A Male Case of Autoerythrocyte Sensitization Syndrome due to Hemoglobin Sensitivity

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*Key Words* A male case Autoerythrocyte sensitization syndrome Hemoglobin sensitivity

*Abstract* The clinical history of a 33-year-old man suffering from an autoerythrocyte sensitization syndrome due to hemoglobin sensitivity is presented.

The rare syndrome of autoerythrocyte sensitization was first described by GARDNER and DIAMOND [1] in 1955 The main clinical feature is the spontaneous appearance either singly or in groups of painful ecchymoses. They are commonly associated with gastrointestinal intracranial, genitourinary bleeding, epistaxis, abdominal pain diarrhea, nausea and vomiting chest pain headache fever and syncopal attacks. This syndrome has occurred only in women [1 3]

In the majority of patients similar ecchymotic lesions can be reproduced by the intradermal injection of autologous and homologous whole blood saline suspension of washed red cells, red cell stroma, or even phosphatidylserine obtained from red cell membranes [1-3] A positive intradermal test obtained with hemoglobin has been rarely reported [4 5]

## *Case Report*

O F (Prot.No. 59 1977). A 33-year-old technician in a hospital from Bursa in the west of Turkey was hospitalized on 3 1 1977 with the main complaint of epi-

Received. May 31 1977 ccepted July 25 1977

3 Hersle, K. and Mobacken, H. Autoerythrocyte sensitization syndrome (painful bruising syndrome). Report of two cases and review of the literature. Br. J. Derm. *81*: 574–584 (1969).
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*Fig 1* Ecchymosis induced by intradermal injection of autologous hemoglobin solution

to find any reported male case with this syndrome in the literature The autoerythrocyte sensitization syndrome due to hemoglobin sensitivity was also rare and reported only a few times [4 5] In this patient there was a close relationship between the occurrence of ecchymotic lesions and severe emotional stresses due to sexual impotence

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L. N. Sussman: **Paternity Testing by Blood Grouping.** C. C. Thomas, Springfield 1976. XVI + 185 pp., US $ 16.50. ISBN 0-398-03523-7

The second edition of Sussman's *Blood Grouping Tests* has been published under the titl *Paternity Testing by Blood Grouping.* Besides the Editor some well-know specialists have contributed and have written individual chapters. The book offers the European reader an excellent survey of the present state of tests in disputed paternity cases in the USA and is w lcome guide for the American medical expert.

L. P. Holländer, *Basel*

J. Lisiewicz: **Hemorrhage in Leukemias.** Polish Medical Publishers, Warsaw 1976. 226 pp. US $ 8.25

Der Autor beginnt das Buch mit einer ausgezeichneten Übersicht der Grundlagen der Hämostase. Er geht ein auf die primäre Hämostase, Interaktion zwischen Thrombozyten und Gerinnungsfaktoren, Fibrinolyse, Thrombose usw. Nachher werden alle Leukämieformen und ihre hämorrhagischen Komplikationen *in extenso* beschrieben. Der Autor beschreibt in allen Fällen mögliche quantitative und qualitative Störungen der Thrombozyten, der Gerinnungsfaktoren und des fibrinolytischen Systems. In einem weiteren Kapitel werden die Nebenwirkungen der antileukämischen Therapie auf den Gerinnungsmechanismus behandelt. Sehr gut präsentiert wird auch der heutige Stand des Wissens über die hämorrhagische Diathese bei paraproteinämischen Krankheiten. Die Literaturangaben umfassen neben wohlbekannten westlichen Referenzen auch uns sonst kaum zugängliche östliche und eine grosse Zahl eigener Arbeiten. Das Buch ist sowohl für hämato-onkologisch wie gerinnungsphysiologisch tätige Ärzte zu empfehlen.

B. Speck, *Basel*

O. Gahrton and X. Y[illegible]aganas: **Progress in Histochemistry and Cytochemistry** vol. 9 No. 1. Quantitative Cytochemistry of Glycogen in Blood Cells. Methods and Clinical Applications. Fischer, Stuttgart 1976. VI + 31 pp., DM 76. *ISBN 3-437 10443-8*

The major part of this publication deals with the technical and methodical aspects of quantitative glycogen measurement in microsystems: dry mass determination by microinterferometry calibrated by microradiography and microspectrophotometry of PAS reaction. A linear correlation between the total extinction and the measured dry mass has been found using glycogen microdroplet reference system. A microfluorometry technique with fluorescent PAS reaction has also been examined with good correlation to the other methods. The specificity of the PAS reaction for glycogen in this system has been assessed by α-amylase digestion removing more than 95% of the PAS-positive material. The same methods are adapted to

# Varia

An Advanced Institute in Methods of Immunological Diagnosis conducted under the auspices of the World Health Organization Collaborating Laboratory for the Serology of Autoimmune Diseases at Wayne State University School of Medicine, will be held June 18–24 1978. The course is to be held on the wooded campus of Cranbrook Educational Community in Bloomfield Hills, Mich.

The course will present an up-to-date survey of current immunological methods applicable to the diagnosis of human immunological diseases.

Category 1 credit for postgraduate medical education has been approved. Deadline for receiving applications is April 15 1978

Further information can be obtained by writing to NOEL R ROSE, MD, PhD, Department of Immunology and Microbiology Wayne State University School of Medicine 540 East Canfield, *Detroit MI 48201* (USA).

## 4 Internationale Arbeitstagung über Lymphknotentumore

Wien, 20.–22. März 1978

Organisation und Auskünfte: Dr P HÖCKER, Ludwig-Boltzmann-Institut für Leukämieforschung, Hanusch-Krankenhaus, Heinrich-Collin-Strasse 50, *A 1140 Wien* (Österreich) Tel 94 21 51/466, 94 41 80

# Erratum

In the article entitled Acquired Aplastic Anaemia in Adults. I A Retrospective Analysis of 40 Cases: Single Factors Influencing the Prognosis by H L HAAK, C A HARTGRINK-GROENEVELD, J G EERNISSE B. SPECK and J J VAN ROOD, published in vol. 58, No. 5 pp. 257 277 (1977), on pages 266 and 267 the legends of figures 1 and 2 were not published completely They should read

*Fig 1* Survival curve for 40 patients suffering from aplastic anaemia (including 11 bone marrow transplantations) – solid line Survival curve for 29 patients given conventional treatment (groups I nd II) – broken line

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# Book Reviews Buchbesprechungen Livres nouveaux

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# Bone Marrow Transplantation Between Mixed Leukocyte Culture Reactive Siblings[1]

K. G. BLUME, K. J. BROSS, R. K. CHILLAR, D. O. FINDLEY, G. OPELZ, R. R. PALADUGU, D. SHARKOFF and J. A. WARNER

City of Hope National Medical Center, Duarte, and
Department of Surgery, University of California, Los Angeles, Calif.

*Key Words.* Bone marrow transplantation · Acute leukemia · Mixed leukocyte culture · Graft vs. host disease

*Abstract.* Bone marrow transplantation from an HLA identical MLC reactive sibling has been performed in patient with acute myelogenous leukemia resistant to drug treatment. Prompt engraftment was documented, however, the patient died of septicemia 34 days after transplant. Clinical manifestation of graft vs. host reaction was mild but was moderately strong expressed at autopsy tissue samples. Recurrence or persistence of leukemia was found at the time of death.

## Introduction

Bone marrow transplantation (BMT) from HLA A, B, D locus identical siblings into patients with aplastic anemia or acute leukemia has become a rather frequently practiced treatment during recent years [7, 9]. However, 2 cases with acute leukemia have been reported in the past in which BMT from HLA-A, B locus identical siblings was done in spite of a positive MLC reaction (D locus nonidentity): 1 patient died 38 days after BMT with septicemia and leukemia [3], whereas another patient is now approximately 2 years posttransplant, free of disease and clinically well without antileukemia treatment [2]. We report here a further case in which BMT was done between HLA A, B identical but MLC-reactive siblings.

[1] This work was made possible in part by support from the Thomas J. Thorley research fund.

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the quantitative determination of glycogen droplets in normal leucocytes after PAS reaction.

The second part deals with the application of the quantitative cytophotometry for glycogen in blood cells of normal individuals, of patients with non-haematological diseases and of patients with diseases of the haematopoietic system (in the last group for example, active CML, low glycogen content of granulocytes with normalization in remission)

In fact the data seem somewhat disappointing from the clinical point of view and do not seem to bring a better diagnostic tool than usual quantitative or semi-quantitative cytochemical analysis of blood cells.

In summary this publication describes very interesting methods for the quantitative cytochemical determination of glycogen in blood and bone marrow cells which may be useful in many research fields but seem hitherto of limited clinical interest.

P. Cornu, *Basel*

N. R. Rose and H. Friedman (eds.) **Manual of Clinical Immunology** American Society for Microbiology Washington 1976. XXV + 932 pp., US $ 20.–
ISBN 0-914826-09 3

This manual is a joint effort by members of the American Society for Microbiology and the American Association of Immunologists The 185 authoritative contributors describe laboratory tests dealing with both humoral and cellular immunological responses of patients. The contents thus include: (A) Tests for humoral components of the immunological response. (B) Tests for cellular components of the immunological response. (C) Immunoassays. (D) Bacterial mycotic and parasitic immunology (E) Viral rickettsial and chlamidial immunology (F) Immunohematology (G) Laboratory examination of patients with allergic and immunodeficiency diseases. (H) Autoimmune diseases. (I) Tumor immunology (J) Transplantation immunology (K) Licensure and certification programs in clinical immunology laboratories.

These sections comprise a total of 124 chapters written by competent investigators. It deals with the practical aspects of the subjects. The general plan of each chapter is usually the following: introduction, clinical indications, test procedures, reagents required, interpretation, and references cited. Though short and limited to essential matters, the various chapters of this comprehensive manual should give access to the necessary information that laboratory directors, technologists and many others may need for performing immunological test in the clinical laboratory It is a useful contribution

A. Micheli, *Genève*

in spite of the reactive MLC, because of increasing difficulty in controlling her disease.

Pretransplant procedures included Broviac catheter placement [1], bone marrow aspiration which showed replacement with blasts containing Auer rods, and lumbar puncture that showed immature granulocytes with Auer rods in the spinal fluid. 25 mg of methotrexate was instilled intrathecally. Conditioning prior to transplant included cyclophosphamide 60 mg/kg on two successive nights and 1,000 rad midline tissue dose total body irradiation (variation of dose 970–1,050 rad along the long axis) with 10 MV X-rays from a linear accelerator on day 0. Bone marrow cells ($7.0 \times 10^8$/kg) were infused thereafter [8].

Early posttransplant complications included pneumonia, congestive heart failure with pulmonary edema, electrolyte imbalance, hemorrhagic cystitis and graft vs. host disease (GVHD). A punch skin biopsy of a diffuse maculopapular rash on day 8 showed intracellular edema of the basal cell layer compatible with grade I GVHD [5]. Concomitant liver function studies were normal. Intermittent diarrhea occurred from day 0 through day 21. GVHD therapy consisted of methotrexate and prednisone; at no time during the course did GVHD appear clinically severe enough to justify treatment with antithymocyte globulin. A day 14 bone marrow aspirate was cellular with XY karyotype in 29/33 cells evaluated indicating successful engraftment; the remaining four cells were inadequate for karyotype identification. Peripheral white cell counts rose to high normal values with the differential showing the majority of cells to be mature neutrophils; no blasts cells were present. The patient's clinical condition was stable at that time of the course. Hepatomegaly was first noted on day 16 with alkaline phosphatase 18.7 B-LIU, total bilirubin 2.7%, SGOT 107 R-F U, SGPT 175 R-F U. On day 23 several episodes of inappropriate behavior were observed. Lumbar puncture, arterial blood gases, electrolytes and renal function studies were normal. Blood ammonia level was 300 μg/100 ml (normal 18–48), total bilirubin 2.7 mg%, direct bilirubin 1.7 mg%, SGPT 59 R-F U, SGOT 36 R-F U. A bone marrow aspirate on day 28 was again cellular without leukemic blasts and 22 of 25 cells analyzed were of XY karyotype. By day 30 she was disoriented to self and surroundings but arousable. Ammonia level was 1,200 μg/100 ml, total bilirubin 4.3 mg%, direct bilirubin 2.4 mg%, SGPT 47 R-F U, SGOT 30 R-F U, alkaline phosphatase 22 B-LIU. Clotting screen and clotting factor analysis were normal. All hepatic metabolized drugs including hyperalimentation were stopped. An electroencephalogram was compatible with toxic or metabolic encephalopathy. Prednisone was discontinued and dexamethasone instituted. A repeat blood ammonia level after 24 h was 600 μg/100 ml. Transaminases and alkaline phosphatase returned to normal. In spite of constant treatment with broad spectrum antibiotics and amphotericin B high fever continued and grade IV coma supervened. Multiple cultures for bacteria, cytomegalovirus, herpes zoster and simplex, toxoplasmosis, and fungi were repeatedly negative. The patient expired on day 34.

Autopsy revealed grade I-II GVHD in skin and liver, while the small intestine showed active lesions consistent with grade III GVHD. The lymph nodes and spleen showed evidence of lymphoid atrophy and in the bone marrow, lymph nodes, and spleen focal leukemia aggregates were detected. In multiple organs, including liver, kidneys, spleen, and lungs, spores and pseudohyphae of candida were found. Post-

## *Case History*

P S. T a 32 year-old Caucasian female was initially diagnosed as having acute myelogenous leukemia in November 1974 M I remission was achieved with cytosine-arabinoside, daunomycin, and oncovin followed by monthly maintenance therapy with cytosine-arabinoside and 6-thioguanine. Her first relapse occurred in July 1975 Subsequent chemotherapy resulted in moderate control of the leukemia but not remission. Multiple hospitalizations were required because of recurrent infections and rising blast counts. She was found to be HLA-A, B identical but MLC-reactive with her male sibling The results of four MLC experiments performed in two institutions between January 1976 and June 1976 using widely accepted techniques [4, 6] are summarized in table I We interpret these results as indicating that the patient and her sibling were HLA D locus incompatible, probably due to a genetic crossover at the D locus. Unfortunately additional siblings were not available to positively prove the crossover BMT was elected in September 1976.

*Table I* Mixed lymphocyte cultures with cells from the recipient, donor and unrelated controls

| Responding cell | Experiment No | Stimulating cell (irradiated) A CPM | A SR | B CPM | B SR | C* CPM | C* SR | D CPM | D SR |
|---|---|---|---|---|---|---|---|---|---|
| A Recipient | 1 | 4,350 | 1 0 | 17 658 | 4 1 | 22,659 | 5.2 | 11,243 | 2.6 |
| HLA 2, W?4/7 W15 | | 1,358 | 1.0 | 3,668 | 2.7 | 4 175 | 3 1 | 4,618 | 3 4 |
| | 3 | 1 472 | 1.0 | 10,100 | 6.9 | 18 913 | 12.9 | | |
| | 4 | 294 | 1 0 | 4 757 | 16.2 | 4,575 | 15 6 | 1,049 | 3 6 |
| B Donor | 1 | 5 176 | 8 8 | 592 | 1 0 | 5,281 | 8 9 | 11 919 | ?0 1 |
| HLA 2, W?4/7 W15 | 2 | 2,140 | 23 0 | 93 | 1 0 | 9 425 | 101.3 | 9,021 | 97 0 |
| | 3 | 6,965 | 4 8 | 1 457 | 1 0 | 11 440 | 7 9 | | - |
| | 4 | 4,562 | 8 6 | 529 | 1 0 | 9 959 | 18 8 | 7,976 | 15 1 |
| C Controls 1 | 1 | 8,356 | 11.3 | 8 798 | 11 9 | 742 | 1 0 | 13,008 | 17.5 |
| 2 | | 4 951 | 13.5 | 9 746 | 26.5 | 368 | 1 0 | 10,015 | ?7.- |
| 3 | 3 | 7 724 | 61.3 | 6,[illegible]12 | 49,3 | 1 6 | 1 0 | | |
| 4 | 4 | 5 194 | 62.6 | 12,726 | 153 3 | 83 | 1 0 | 3 756 | 45.3 |
| D Controls 5 | 1 | 8,548 | 36.7 | 12,605 | 54 1 | 4,230 | 18.2 | [illegible]33 | 1 0 |
| 6 | | 3 698 | 11 6 | 9 970 | 31 4 | 18,777 | 59 1 | 318 | 1.0 |
| | 3 | | – | | | | | | |
| 7 | 4 | 5,890 | 77.5 | 11,218 | 147 6 | 7,816 | 102.8 | 76 | 1 0 |

Experiment 1 and 2 were performed following the method of SENGAR and TERASAKI [6], experiments 3 and 4 were done according to HARTZMAN *et al* [4]

in spite of the reactive MLC, because of increasing difficulty in controlling her disease.

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| 2 | 2 | 4,951 | 13.5 | 9 746 | 26.5 | 368 | 1 0 | 10,015 | 7.2 |
| 3 | 3 | 7 774 | 61.3 | 6,212 | 49,3 | 126 | 1 0 | | |
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8 Thomas, E. D. and Storb, R. Technique for human marrow grafting. Blood *36* 507–515 (1970).
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mortem cultures of brain, kidney lung and spleen grew various bacterial organisms including staphylococcus aureus (coagulase negative), *Enterobacter agglomerans, Pseudomonas aeruginosa* and *Flavobacterium* sp Cultures of the Broviac catheter for bacteria and fungi were negative The immediate cause of death appeared to be progressive coma culminating in respiratory and cardiac arrest.

## Discussion

A bone marrow transplantation has been performed in an end stage leukemia patient from an HLA A B identical MLC reactive donor Clinical signs of GVHD were discrete whereas histological findings on postmortem specimens were more pronounced The fact that about 90% of the bone marrow cells were of donor type but residual patchy leukemia areas were found at autopsy can probably be interpreted as engraftment of donor bone marrow and persistence of focal leukemic tissue a recurrence of leukemia in donor cells is theoretically possible.

Because this patient succumbed to overwhelming septicemia rather early in her posttransplant course the ultimate role of a positive MLC reaction cannot be fully evluated from this case although our findings are in agreement with those of others [2 3] that engraftment can be achieved in spite of an MLC barrier It may be that a positive MLC does contribute to an increased susceptibility to infections. However septicemia and GVHD are also frequently observed complications in MLC-identical transplants.

A noteworthy feature in this case is also the finding of persistence or recurrence of leukemia in the presence of MLC incompatibility and moderately severe GVHD showing the lack of a graft vs leukemia effect even across an MLC barrier

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# A New Case of Gamma Heavy Chain Disease

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*Key Words* Franklin's disease γ-Heavy chain disease γ-Heavy chain disease proteins Immunoglobulins Immunoblastic sarcoma Malignant lymphoma Monoclonal gammopathy

*Abstract* The first case of γ-heavy chain disease described in Spain is here reported. The patient, a 36-year-old woman, presented fever enlarged regional lymph nodes, and hepatosplenomegaly without bone marrow abnormalities but with lymphopenia. Serum electrophoresis did not disclose any M-component. The abnormal γ-chain protein had an $\alpha_1$ globulin mobility and was immunochemically related to the Fc fragment. It belonged to the IgG 4 subclass, its molecular weight was about 60,000 Proteinuria was minimal but the electrophoresis of concentrated urine showed a homogeneous peak of α-globulin mobility constituted by the γ-chain fragment. Biopsy of an axillary lymph node disclosed features of immunoblastic sarcoma The course was malignant, resulting in death in 8 months.

## *Introduction*

The heavy chain disease (HCD) concept arose in 1964 when Franklin *et al* [15] and Osserman and Takatsuki [24] published, in 5 patients with lymphoproliferative processes, the finding in the serum and the urine of a new type of paraproteinemia consisting of the presence of a homogeneous population of γ-chain fragments being devoid of light chains and immunochemically related to the Fc fragment produced by papain digestion of IgG

In 1968 Seligmann *et al* [32] published the first case of the α-HCD

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type which seems to be rather more frequent than the γ-HCD for since then till now more than 63 cases [17 18 28] have been described.

In 1970 the μ-HCD variety was also described in 2 patients with chronic lymphatic leukemia [1 4 8, 12]

The δ- and ε-HCD types have not been described yet. The γ-HCD is rare: only 30 cases have been described from 1964 until 1973 [13] According to our information, at least 6 more cases [3 19 21b, 25 31 32] have been published from the former date. It seems to be clear that the clinical features, although considerably diverse, generally belong to the malignant lymphoma pattern.

On the other hand, the clinical diagnosis of the γ-HCD is, by defintion impossible and must be always based on a meticulous study of the serum and urine proteins, with the idea of detecting the presence of γ-chain fragments lacking light chains and immunochemically related to the Fc fragment of IgG

The purpose of this report is to present the clinical, pathological and immunochemical features of the first case described in our country

## *Case Report*

*Clinical and Pathological Data*

J Dou 36-year-old housewife, married, childless, without any particular personal or family history of the disease She was admitted to the Department of Medicine on May 8th, 1974 as a result of having noted the following signs and symptoms 15 days prior to admittance left side facial and cervical tumefaction, dysphagia, irritating cough, dyspnea, edema of the left arm, fever asthenia, anorexia and weight loss of 4 kg. She was also suffering from aphonia which had begun 2 months before.

Physical and radiological examination revealed swollen axillary and supraclavicular lymph nodes as big as walnuts, hard, not fixed and painless; massive effusion in the left pleural cavity with contralateral displacement of mediastinum, signs of mediastinic compression with tumefaction of the face, neck and left arm, facial cyanosis, jugular distention, orthopnea, tachycardia (130 bpm) without murmurs, temperature 38.8 °C, moderate hepatomegaly radiological splenomegaly There were no osteolytic lesions. There was no swelling of the uvula and palatal edema.

Laboratory data (table I): 1 700 ml of serohematic liquid were extracted from the left pleural cavity

Bacteriological examination and culture were negative. Cytological examination of pleural liquid revealed the presence of numerous lympoid-type cells, atypical, which were listed as being of lymphosarcomatous aspect. The bone marrow examination was within the normal limits. An axillary lymph node biopsy revealed that the normal architecture of the lymph node was effaced and the parenchyma was in-

*Table I* Laboratory data of patient J Dou.

| | 1st admission (10–5–74) | 2nd admission (15–8–74) | 3rd admission (25–10–74) |
|---|---|---|---|
| ESR mm/h | 40 | 67 | 125 |
| Hematocrit, % | 36 | 36 | 79 |
| Hemoglobin g/dl | 14 0 | 11 0 | 9 0 |
| Leucocytes/µl | 6,800 | 5 400 | 4,500 |
| Neutrophils, % | 88 | 80 | 78 |
| Lymphocytes, % | 8 (544/µl) | 18 (79[illegible]/µl) | [illegible]0 (900/µl) |
| Monocytes, % | 2 | 2 | 2 |
| Platelets | WNL | WNL | WNL |
| Bone marrow | WNL | WNL | increase of |
| Coagulation tests | WNL | W L | erythroid series |
| Glucose, mg/dl | 68 | 86 | 8[illegible] |
| Urea, mg/dl | 75 | 30 | 35 |
| Uricemia, mg/dl | 4 8 | 2.7 | [illegible]1 |
| Creatinine, mg/dl | 0.95 | 0 90 | 0.82 |
| Total lipids, mg/dl | 625 | 518 | 532 |
| Cholesterol, mg/dl | 168 | 135 | 130 |
| Bilirubin, mg/dl | 0.50 | 0 40 | 0.50 |
| SGOT URF | 27 | 40 | 56 |
| SGPT URF | 25 | 39 | 40 |
| Alkaline phosphatase, mU/ml | 30 | | 38 |
| Total serum protein, g/l | 66 | 61 | 57 |
| Serum electrophoresis | | | |
| Albumin % | 52.4 | 50.0 | 44.7 |
| $\alpha_1$-Globulin, % | 4 9 | 5.3 | 7 2 |
| $\alpha_2$-Globulin % | 19 6 | 20 7 | 23 9 |
| β-Globulin | 14 7 | 14.5 | 14 5 |
| γ-Globulin % | 8 1 | 9 5 | 9 3 |
| Urine proteinuria, g/24 h | 0.17 | 0.13 | 0.16 |
| Electrophoresis of concentrated urine | | | |
| Albumin % | 13 0 | 10.0 | 14 0 |
| $\alpha_1$-Globulin % | 0 0 | 0 0 | 0 0 |
| $\alpha_2$-Globulin % | 85 0 | 86 0 | 83 0 |
| β-Globulin % | 2.0 | 4 0 | 3 0 |
| γ-Globulin, % | 0 0 | 0 0 | 0.0 |
| Rheumatic serology | negative | | |
| Syphilis serology | negative | | |
| Paul-Bunnell test | negative | | |
| Toxoplasma tests | negative | | |
| Antibodies to erythrocytes | negative | | |

*Table I* (continued)

| | 1st admission (10-5-74) | 2nd admission (15-8-74) | 3rd admission (25-10-74) |
|---|---|---|---|
| Antinuclear antibodies | negative | | |
| Antimitochondrial antibodies | negative | | |
| Antitissue antibodies | negative | | |
| Coldagglutinins | negative | | |
| Cryoglobulins | negative | | |
| Chromosomes studies | | normal | |

WNL = Within normal limits.

vaded by highly cellular tissue with predominance of large cells (immunoblasts) with pyroninophilic cytoplasm, irregular nuclei and nucleoles. Some binucleated cells were discernible. There was an important vascular component, limbs with swollen endothelial cells. The capsule and perinodular tissue were not invaded. The diagnosis was immunoblastic sarcoma (fig. 1).

The EKG showed sinusal tachycardia and diffuse disturbance of the repolarization. The laryngoscopic examination disclosed recurrent left paralysis. The planigraphy confirmed the presence of mediastinal lymph nodes in both pulmonary hilums and bilateral paratracheal lymph nodes as well.

On May 18th 1974 cytostatic therapy was started, consisting of cycles of 1 week's duration at intervals of 15 days, according to the following distribution: 2 days of vincristine (1.4 mg/m²) followed by 5 days with cyclophosphamide (400 mg/m²). The cytostatic therapy showed rapid and efficacious results.

The patient left the hospital without any symptomatology on June 26th 1974.

In spite of the cytostatic therapy the above-described clinical pattern repeated itself and the patient was admitted to hospital on two other occasions; in August 1974 and October 1974. During the last admission the appearance of 4–5 papuloerythematous cutaneous lesions in the front area of the thorax was confirmed. Histological examination revealed dermical infiltrate consisting of atypical lymphoids and histiocytoid cells as well as small number of plasmocytoid cells.

The patient died at home on December 20th 1974, with clinical pattern of respiratory insufficiency. An autopsy could not be carried out.

*Protein Studies*

*Material and methods.* The serum (which had natrium azid added to it) was kept in cold storage at temperature of –25 °C until it was used. The 24-hour urine samples, collected with natrium azid were concentrated 80–100 times through dialysis with Visking membrane against polyethyleneglycol (Carbowax). The serum and concentrated urine electrophoresis was carried out on cellulose acetat (Cellogel, Chemetron) according to standard procedures. The immunoelectrophoresis ana-

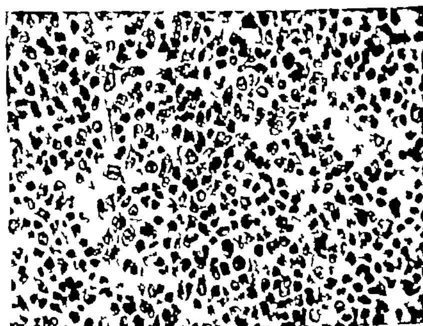


*Fig. 1* Histological pattern of axillary lymph node. Immunoblastic sarcoma. HE. × 450.




*Fig. 2* Electrophoresis of serum and urine. PU = Patient's urine. PS = patient's serum. An M-component in the $\alpha_2$-globulin region is observed in the electrophoresis of urine, but not in the serum.

lyses were done on 1 / agarose-gel (Miles Laboratories) in barbital buffer (ionic strength 0.50 pH 8.4) with some personal modifications to the classical Grabar method. The whole antihuman, anti-IgG (monospecific to $\gamma$-chains) anti-IgG (containing a tibodies to both heavy and light chains), anti-Fab/IgG anti-Fc/IgG and anti-Fd/IgG antiserums were obtained from Behringwerke. The anti Ig G + IgA + IgM anti-$\varkappa$ and anti-$\lambda$ antisera were obtained from Grenlab.

Quantitative determinations of immunoglobulins serum were carried out by simple radial immunodiffusion according to the M ncini method, using M-partigen (Behringwerke).

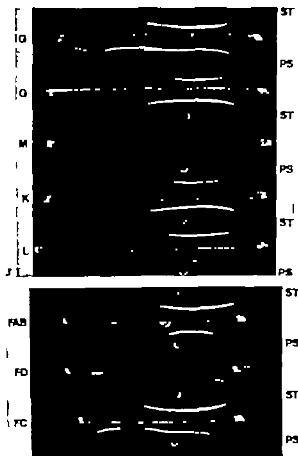



*Fig 3 4* Immunoelectrophoresis of patient serum. With anti-IgG and anti-Fc/IgG antiserum a clear double arc is observed due to the presence of γ-heavy chain fragments. With anti-κ, anti-λ anti-Fd/IgG and anti-Fab/IgG the double arc did not appear. ST = Normal serum, PS = patient serum. IG = anti-IgG-IgA-IgM. G = anti-IgG. M = anti-IgM. K = anti-κ. L = anti-λ. FAB = anti-Fab/IgG. FD = anti-Fd/IgG. FC = anti-Fc/IgG

The subclass of IgG was typed by FRANÇOISE DANON MD (Laboratory of Immunochemistry Prof M SELIGMANN, Hôpital Saint-Louis, Paris). Gel filtration chromatography of the patient's serum and urine, was performed on 2.5 × 70 cm columns, packed with Sephadex G-200 superfine and Sephadex G-75 superfine (Phar




*Fig. 5.* Immunodiffusion for IgG determination according to the Mancini method. A clear double circle is seen in the precipitin area of the patient's serum due to the presence of γ-heavy chain fragments. Wells 7 and 8 = Patient's serum, wells 3–6 = normal serums.

macia Fine Chemicals) equilibrated with 0.1 M Tris-HCl containing 1 M NaCl, pH 8.0.

The protein content of eluates was checked in a Zeiss Spectrophometer (280 nm). The eluates were concentrated by dyalisis through Collodion membranes (Sartorius) against vacuum.

*Results.* Total protein concentration in the serum was 66 g/l (table I). In the serum electrophoresis the presence of any homogeneous peak (M-component) was not disclosed, but an increase of $\alpha_1$ globulin and a decrease of γ-globulin were seen (fig. 2).

Proteinuria was 0.17 g/l (table I). The electrophoresis of concentrated urine showed the presence of a homogeneous peak (M-component) which had an electrophoretical mobility of α-globulin and signified more than 80% of the total protein content (fig. 2, table I).

Immunoelectrophoresis of the serum revealed the presence of an anomalous precipitin arc for the IgG and this could be seen with an anti IgG IgA IgM as well as with a monospecific anti IgG antiserum.

The IgG precipitin arc appeared with a double arc with a partial identity reaction: the abnormal arc had an -globulin mobility and the cathodal arc corresponded to the normal IgG. The nomalous precipitin arc was not reproduced with anti κ, anti λ, anti-Fab/IgG, anti-Fd/IgG antiserums, but it did appear with an anti-Fc/IgG antiserum (fig. 3, 4).

Immunoglobulin levels in the serum were: IgG 480 mg/l, IgA 22 mg/l, IgM 10 mg/l. The precipitin area of IgG showed a clear double circle (fig. 5). The smaller was also more intense (which would correspond to the normal IgG) and valued individually this supposed 58 mg/l. (The quantitative value of the abnormal γ-chain fragment can obviously not be considered as n exact amou t but only as an indicative figure of its high concentration, since an IgG fragment is considered here and it does not have the same diffusion abil ty as an intact immunoglobulin which is used as standard in the Mancini method.)

The urine immunoelectrophoresis revealed the presence of a considerably homogeneous precipitin arc with n anti-IgG antiserum which corresponded to the M-component detected by electrophoresis.

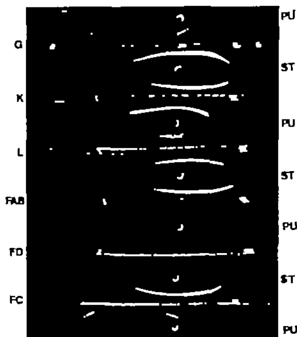



*Fig 6* Urine Immunoelectrophoresis. The M-component is constituted by γ-heavy chain fragments, it reacts with anti-IgG and anti-Fc/IgG antisera but not with anti-κ, anti-λ, anti-Fab and anti-Fd antisera.

As occurred with the serum, this arc was not reproduced with anti- anti-λ, anti-Fab/IgG anti-Fd/IgG but it reacted with an anti-Fc/IgG antiserum (fig. 6). The abnormal γ-chain fragment was an IgG 4. The elution diagrams of serum and urine column chromatography on Sephadex G-200 can be seen in figur 7 and 8. The immunoelectrophoresis of chromatographical fractions of the serum showed that the normal IgG was eluted in the peak called F2, corresponding to the substances with molecular weight of 7S.

Contrary to this, the abnormal IgG fragment was contained in the peak called F3, corresponding to the substances of molecular weight 3.5S. Results for the urine were the same: the M-component was eluted in the three subfractions called F3' F3'' F3'''.

The F3 chromatographical fractions on Sephadex G-200 of the serum and urine were rechromatographed on Sephadex G-200 and Sephadex G-75 to verify the a- ay which indicated that the abnormal γ-chain fragment had molecular weight of about 60,000.

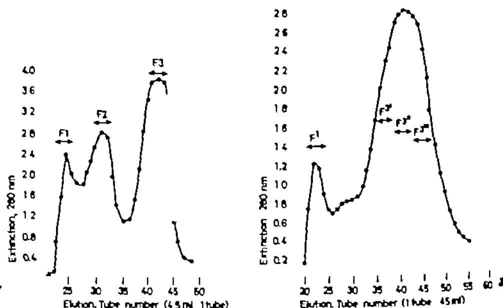



*Fig 7* Column gel chromatography on Sephadex G 200. Patient s serum.
*Fig 8* Column gel chromatography on Sephadex G 200. Patient s urine.

## Comments

In order to compare the clinical biological and pathological data found in our patient with the data of other cases, we include a summary of the clinical and laboratory features of 24 patients with γ-HCD carried out by FRANGIONE and FRANKLIN [13] (table II)

In the right hand column the presence (yes) or the absence (no) are indicated for patient J Dou from each one of the listed data

The lesions described in the different patients may be classified into three groups. The largest group consists of those in which a simultaneous infiltration of lymphocytes and plasma cells existed very often accompanied by eosinophils and reticulum cells without Sternberg cells [6 9 10 21a, 35] This histological pattern was difficult to classify

In some cases no features of malignancy existed In several of these cases the diagnosis was given as an atypical Hodgkin s disease One could wonder if such descriptions are morphologically within the so-called immunoblastic lymphadenopathy [23] or even whether there is any connection. In the smallest group, those cases in which histological atypia was a marked characteristic, i e with the diagnosis of reticulum cell sarcoma

*Table II* Clinical and laboratory features of [illegible]4 patients with γ-HCD summarized by Frangione and Franklin [10] in 1973, and of the patient J. Doo.

| | Frangione and Franklin revision | Patient J. Doo. |
|---|---|---|
| Sex | 19 M 11 F | F |
| Race | 22 White, 5 Black 3 Japanese | White |
| Age, 18–36 years | (four under 40 17 18, 21 37) | 36 |
| *Clinical features* (24 patients) | | |
| Onset | | |
| Insidious | 14 | no |
| Rapid | 10 | yes |
| Lymphadenopathy | 17 | yes |
| Anemia | 13/22 | no |
| Hepato and/or splenomegaly | 9/22 | yes |
| Fever | 13/22 | yes |
| Lymphadenopathy and fever | 12/22 | yes |
| Course | | |
| Lymphadenopathy | 19/21 | yes |
| Local | 5/21 | yes |
| Generalized | 14/21 | no |
| Waxing and waning | 5/22 | no |
| Splenomegaly | 16/23 | only radiological |
| Hepatomegaly | 16/23 | yes |
| Swollen erythematous palate | 8/21 | no |
| Fever | 15/20 | yes |
| Infections | 20/20 | no |
| X-rays of bone negative | 20/20 | yes |
| Cause of death | | |
| Sepsis | 10 | no |
| Malignancy | 6 | yes |
| (?) or alive | 8 | |
| Duration | 5 weeks to 5 years | 8 months |
| *Laboratory features* (21 patients) | | |
| Elevated ESR | 5/8 | yes |
| Anemia | 13/22 | no |
| Leukopenia with relative lymphocytosis | 13/21 | lymphopenia |
| Plasma cell leukemia | [illegible]/21 | no |
| Thrombocytopenia | 9/20 | no |
| Eosinophilia | 6/20 | no |
| Hyperuricemia | 8/14 | no |

*Table II* (continued)

| | FRANGIONE and FRANKLIN revision | Patient J. Dou. |
|---|---|---|
| Bone marrow | | |
| 1 Plasma cells | 1 | no |
| Lymphocytes | | no |
| 3 Eosinophils in addition to 1 and | 4 | no |
| 4 Eosinophils only | 1 | no |
| 5 Normal | 4 | yes |
| Serum spike | | |
| > 1 g/dl | 9 | |
| < 1 g/dl | 7 | – |
| Negative | 1 | – |
| Not known | 7 | – |
| Hypo-γ-globulinemia | 13/16 | yes |
| Urine proteinuria | | |
| > 1 g/24 h | 4 | no |
| < 1 g/24 h | 7 | |
| Trace | 8 | yes |
| Not known | 5 | – |
| Bence-Jones protein negative | 23/24 | yes |

[20 21] would be classified It is probable that these cases correspond to immunoblastic sarcoma like the case we handled [22]

If we take into account the possibility of transition from an immunoblastic lymphadenopathy to an immunoblastic sarcoma [23] it becomes significant to weigh the possibility of a chronological relationship between the two groups mentioned In this way it is interesting to comment that in various cases the diagnosis was carried out in patients who had previously been subject to so-called autoimmune disease rheumatoid arthritis [14 35] Sjögren's syndrome [13] myastenia gravis [21] hemolytic anemia [34] lupus erythematosus [30]

This could be considered somewhat more than sheer chance, especially if one takes into account the clinical observations which would make one suggest a possible relationship between a prolonged antigenic stimulation and the development of a nonneoplastic lymphadenopathy and the transition from this to a lymphoplasma-reticulum proliferative malignant disease [23 27]

A third group would consist of those cases in which a predominant infiltration of plasma cells were found, lacking in atypia or even without it, but with malignant features due to effacing of the normal lymph node structure [24 26] It should be noted that 2 patients died from plasma cell leukemia [30 34]

As regards the immunochemical diagnosis of the abnormal protein, it must be stressed that in spite of its high concentration, the electrophoresis of the serum did not disclose any M-component, because the abnormal γ-protein had an $\alpha_2$-globulin mobility and was confused with the usual aspect of this fraction, although it was increased. The insufficiency of electrophoretical methods exists in the majority of α- and μ-HCD cases [1 2, 4 8, 12, 17 18, 29] However in all the γ-HCD cases, except for a few [6, 30] the serum electrophoresis gave rise to the disclosure of an M-component of a β/γ mobility or an abnormal broad band of β/γ mobility [13] The elimination of the abnormal protein through urine is virtually constant, not withstanding a slight proteinuria, as in our case, and thus the opposite of α- and μ-HCD cases. Immunoelectrophoretical analysis of protein Don. showed that this protein only carried antigenic determinants of the Fc fragment of IgG lacking Fab, Fd, κ- and λ-chain determinants. From 27 of γ-HCD proteins typed, protein Don. constitutes the second IgG 4 γ-HCD protein which we know [13 19]

The chromatographic behavior of native protein Don. indicated that the molecular weight was around 60,000 The molecular weight of the other γ-HCD proteins studied in their native state varies from 20,000 [19] to 80,000 [34], although it is generally around 50,000 [11, 15 21, 24 34] In the cases where the γ-HCD proteins were reduced and alkylated [11 15 26, 34] it was demonstrated that the native protein constituted a dimer of two Fc fragments whether linked by disulfide bonds or not.

It has been shown that the abnormal protein is not a product of an extracellular proteolysis, but a product of an aberrational and incomplete synthesis of the γ-chains from the proliferation B cells which, at the same time, are unable to synthesize light chains [5 11] The structural studies of some γ-HCD proteins have disclosed that the molecular anomaly consisted of an *internal deletion of the γ-chain sequence* [7 11, 14 16, 33] The variability in the size of the deletion explains the heterogeneity of the different γ-HCD proteins as regards molecular weight, physicochemical properties and immunochemical features.

Obviously the knowledge of the structure of these abnormal proteins is of considerable theoretical interest since knowing the mechanism of

their deletion, as well as the inability of the light chain synthesis in the cells which produce the abnormal protein, new ideas and hypotheses may be conceived on the normal genetic control of the immunoglobulin synthesis.

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# Acquired C1 Inhibitor Deficiency in Essential Cryoglobulinemia and Macrocryoglobulinemia

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*Key Words.* Complement Acquired C1-inhibitor deficiency Essential cryoglobulinemia Waldenström's disease Immune complexes $^{125}$I C1q binding activity

*Abstract.* In 5 patients with mixed cryoglobulinemia, 3 with Waldenström's disease and 2 with essential cryoglobulinemia, C1-inhibitor (C1-INH) deficiency was discovered. The complement profile was characteristic of the acquired type: the total hemolytic activity and the early components were reduced, C3 was diminished in 1 patient only C5 and C9 were normal or elevated. 1 patient with Waldenstrom disease and 1 with essential cryoglobulinemia experienced episodes of angioedema. Circulating immune complexes were found in all patients' sera by the $^{125}$I-radiolabelled C1q binding activity (C1q BA) test. The values of the C1q binding activity were correlated with the depletion of the early complement components and that of C1 INH. 4 patients showed circulating 7S IgM. Our data support the hypothesis of complement activation by the cryoprecipitating immune complexes; the C1 INH depletion is secondary to its consumption following C1 activation.

A congenital deficiency of the inhibitor of the first complement component C1 inhibitor (C1 INH) is characteristic of hereditary angioedema (HAE) [6] Until now only a few cases of acquired C1-INH deficiency have been reported in lymphoproliferative disorders with serum immunoglobulin abnormalities, in cryoglobulinemias and in systemic lupus erythematosus [1 4 5 8, 10, 17] In these patients the clinical pattern of angioedema may be lacking. The complement profile is typical.

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consumption of the early components of the classical pathway with normal levels of C3 and C5-C9 Frequently a low molecular weight IgM (7S IgM) has been suggested as the basis of such data. These findings should be strengthened by the evidence that the 7S IgM-containing sera can deplete the first complement components when incubated with normal serum [1 8]

It is the purpose of this paper to investigate the correlation between the CI INH depletion, the serum 7S IgM and the level of circulating immunocomplexes in 5 patients with mixed cryoglobulinemia and CI INH deficiency· 3 with Waldenström s disease and 2 with the syndrome of MELTZER and FRANKLIN [13]

## Materials and Methods

*Patients* Table I shows the clinical data of our patients. 2 of these experienced episodes of angioedema. In no case was there a family history of HAE nor were the symptoms present in youth.

*Serologic studies.* Only one specimen of blood from each patient was available except for the case 1 who was followed for 4 months.

*Cryoglobulins.* Blood was collected into plastic syringes prewarmed at 37 °C. Serum was separated at 37 °C and the cryoglobulinemic fraction was precipitated

*Table I* Clinical description of 5 patients with C$\bar{1}$ INH deficiency

| Case | Patient | Age | Sex | Diagnosis | Cryoprecipitate (cryocrit) | Clinical features of angioedema |
|---|---|---|---|---|---|---|
| 1 | S. M | 57 | M | macroglobulinemia | IgG k λ<br>IgM k (77%) | – |
| 2 | C. G | 55 | M | macroglobulinemia | IgG k λ<br>IgM k (90%) | – |
| 3 | C. M. | 59 | F | essential cryoglobulinemia | IgG k λ<br>IgM k (3 7%) | attacks involving the face and lips |
| 4 | C. B. | 57 | F | macroglobulinemia | IgG k λ<br>IgM k (31 %) | attacks involving the tongue and laringeal mucous membranes |
| 5 | Z. G | 52 | F | essential cryoglobulinemia | IgG k λ<br>IgM k (20 %) | – |

and recovered according to MELTZER and FRANKLIN [13]. Isolated and washed cryoglobulins were resuspended in a volume of 0.15 M NaCl equal to initial serum volume. The amount of cryoglobulin sera was evaluated by cryocrit, indicating the volume occupied by the cryoprecipitate, expressed as volume percentage of whole serum after centrifugation at 4 °C for 10 min at 700 g of the serum stored at 4 °C for 72 h.

*Immunoelectrophoresis* was carried out following the micromethod of SCHEIDEGGER [18].

*Complement studies.* Blood was allowed to clot at room temperature; serum was either examined directly or kept at 70 °C. Total hemolytic complement activity was determined according to the method of LACHMANN et al. [11]. Fresh normal human serum (NHS) values ranged from 800 to 1 400 hemolytic U/ml (HU/ml). C4, C5 and properdin factor B(C3PA) activity was estimated by the 'lysis plates' assay [11] and C3 was titrated by fluid phase method [11]. C1q, C9 and C1-INH concentrations were determined by simple radial immunodiffusion in agarose 1.5%, veronal 0 075 M, EDTA 0.01 M, pH 8.2; the monospecific antisera were purchased from Behringwerke. The titers of all complement components and C1-INH were expressed as percentage activity or concentration of pool of fresh NHS.

*Circulating immune complexes determination.* C1q was isolated from fresh NHS using relative salt concentrations of 0.04 and 0 078 M NaCl, respectively for the first and second precipitation steps [20, 21]. The purified C1q was radiolabelled with $^{125}$I (Radiochemical Centre, Amersham, England) by lactoperoxidase (Calbiochem, Calif.) [9 14]. The specific activity was 1 μCi/μg. The $^{125}$I C1q binding activity test was performed in the sera as described [24].

*Low molecular 7S IgM* were demonstrated by double immunodiffusion of sera in 4% polyacrylamide gel [1].

## *Results*

### *Complement Assays*

The results are shown in table II. The levels of the total hemolytic activity and of the fractions C1q and C4 are markedly reduced in all patients, while the serum concentration of C3 was normal or elevated in 4 and diminished in 1 properdin factor B was markedly reduced in the 1 patient with the lowest level of C3 C5 was low or normal C9 was normal or significantly elevated in 1 patient (case 2). C1-INH was always reduced. in 4 patients it was severely depleted.

### *C1q Binding Activity*

This test was positive in all our patients (table II). The C1q BA of each sample was closely related to the depletion of C1 INH and the reduc tion of C1q and C4 No relation was shown between the C1q BA and the level of C3 C5 C9 C3PA or the total hemolytic activity Figure 1 shows

*Table II* Serum complement values and circulating immune complexes (C1q BA)

| Case | Patient | CH 50 | C1q | C4 | C3 | C5 | C9 | Properdin factor B | CI-INH | C1q BA |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | S. M. | 476 | 50 | 24 | 95 | 50 | 130 | 84 | 0 | 64 |
| 2 | C. G | 400 | 24 | 22 | 85 | 57 | 170 | 65 | 10 | 71 |
| 3 | C. M. | 280 | 44 | 14 | 175 | 90 | 110 | 84 | 28 | 68 |
| 4 | C. B | 400 | 55 | 25 | 62 | 50 | 80 | 80 | 55 | 50.5 |
| 5 | Z. G | 200 | 24 | 0 | 38 | 60 | 85 | 34 | 25 | 61 |

CH 50 = HU/ml (normal levels 800–1 400 HU/ml) C1q CI INH, C4 C3 C5, C9 Properdin factor B = percent value compared with fresh NHS (normal value > 60 ) C1q BA = percent value compared with TCA positive control (where all the protein is acid precipitated) – normal value < 6%.

*Table III* Circulating 7s IgM in 5 patients with positive $^{125}$I-labeled C1q binding activity

| Case | Patient | Diagnosis | CI INH | Cryocrit | C1q BA | 7s IgM |
|---|---|---|---|---|---|---|
| 1 | S. M. | macroglobulinemia | 0 | 77% | 64 | + |
| 2 | C. G | macroglobulinemia | 10 | 90% | 71 | + |
| 3 | C. M. | essential cryoglobulinemia | 28 | 3 7% | 68 | − |
| 4 | C. B | macroglobulinemia | 55 | 31% | 50.5 | + |
| 5 | Z. G | essential cryoglobulinemia | 25 | 20% | 61 | + |

the relationship between the levels of CI INH, C4 the anticomplementary activity (C1q BA) and the cryocrit in serial specimens obtained in case 1 during the follow-up when the patient was treated with plasmapheresis. The greater the anticomplementary power is, the lesser the CI INH level. These characteristic data suggest the hypothesis of an acquired CI INH deficiency

*7S IgM*

In all the patients with Waldenström s disease and in one with essential cryoglobulmemia, low molecular weight IgM (7S IgM) were found these same patients showed the highest cryoprecipitate levels (table III)

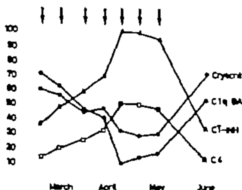



*Fig 1* Patient S.M. (case 1). △ = C1-INH □ = C4 percent value compared with fresh NHS. O = C1-qBA. percent value compared with TCA positive control. ● = cryocrit value; Arrows = cicles of plasmapheresis.

*Discussion*

The complement profile found in our patients, i.e., severe reduction of the level of C1 INH, consumption of the early components and reduction of the total hemolytic activity (CH50), with sparing of the later ones and occasional augmentation of C9 and the absence of clinical signs of HAE both in the family history and in the youth, are likely to indicate an acquired C1 INH deficiency [1 5 8, 10, 17] In this regard it is important to stress the reduction of C1q found in all our patients. This feature is incompatible with the HAE where the C1q concentration and the whole C1 activity are always normal.

Only in case 5 was a reduction of C3 detectable and this parallels a consumption of properdin factor B A reduction of C3 was been previously described only in a case of single cryoglobulinemia with cold urticaria [4] The impairment of C3 and properdin factor B could be justified by the triggering of the C3b amplification loop however a direct complement activation via the alternate pathway could not be excluded. The presence of 7S IgM in our patients with Waldenström's disease is not an uncommon finding [2, 19] More interesting is this result in 1 of the 2 patients with essential cryoglobulinemia with monoclonal IgM K, also in primary cold agglutinin disease low molecular weight IgM have been found [2]

It has been previously demonstrated [1 8] that normal sera, when incubated with abnormal 7S IgM, display an acquired C1 INH deficiency

This suggest a CI INH depletion subsequent to the Cl activation, through an unproven mechanism, IgM 7S dependent [1 8] However some data do not seem to support this hypothesis HAUPTMAN *et al* [8] found an increase in CI INH level during a posttransfusional hepatitis despite the presence of the abnormal immunoglobulin at a steady titer A lowered hemolytic activity of purified 19 S cold agglutinins, after reduction to 7S subunits has also been reported [3] Furthermore, in our study case 3 displayed a CI INH depletion in the absence of low molecular weight IgM

In our patients sera, immune-complexes were detected by $^{125}$I-radiolabelled C1q BA this test allows the direct quantitation of C1q fixing complexes in native serum and it is highly specific [22] The C1q BA was correlated with the depletion of the early complement components, particularly with the C1q and CI INH level It seems possible to suggest that this pattern is indicative of an immunologic mechanism for C1 fixation due to circulating complexes the CI INH reduction is probably secondary to this activation Positive results with this test in all patients with lymphoproliferative diseases were also obtained by HAUPTMANN *et al* [8] on the contrary CALDWELL *et al* [1] did not find circulating immune complexes by a variety of techniques (C1q or rheumatoid factor precipitation, analytical ultracentrifugation, immunodiffusion analysis of fractions from sucrose density gradients) whose sensibility is of a lesser degree when compared with the C1q BA test.

The serum complement monitoring in the patient we could follow over a 4-months period during plasmapheresis treatment, seems to confirm this hypothesis: every time that C1q BA was elevated, CI INH and C4 levels were low on the other hand, when the anticomplementary activity failed, CI INH and C4 were raised This behavior is independent of the continuous 7S IgM presence Considering the positive $^{125}$I C1q BA test as evidence of circulating immune complexes, our data support the hypothesis that the observed pattern of interaction in the studied sera is determined by the antigen-antibody nature of the mixed cryoglobulinemias. Furthermore, ROTHER *et al* [16] and MÜLLER *et al* [15] came to the conclusions that mixed cryoglobulins activate the first complement components in the same way as immune complexes and aggregated immunoglobulins do

Our results are, finally in agreement with those recently reported by DAY *et al* [5] who described a patient with chronic lymphocytic leukemia and circulating cold precipitable immune complexes, consisting of lym-

phocyte membrane antigens and IgM antilymphocyte antibody suffering of episodes of angioedema with the typical complement profile of acquired C1 INH deficiency

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# Acid Hydrolases in Normal B and T Blood Lymphocytes[1]

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City of Hope National Medical Center, Duarte, Calif.

*Key Words.* Acid Hydrolases · Biochemistry · B and T lymphocytes · Acid phosphatase · β-Glucuronidase · α-Mannosidase

*Abstract.* B and T lymphocytes were separated by means of the spontaneous sheep red blood cell rosette formation technique from 3 normal donors. The following acid hydrolases were biochemically determined on separated B and T lymphocytes: acid phosphatase, β-glucuronidase, β-galactosidase, β-hexosaminidase, α-arabinosidase, α-galactosidase, α-mannosidase, α-glucosidase, and pH 4.0 and pH 5.0 β-glucosidase. The activities of most of the acid hydrolases including acid phosphatase and β-glucuronidase were found to be slightly decreased in B lymphocytes when compared to T lymphocytes. However, α-mannosidase activity was found to be significantly higher in the B lymphocytes than in the T lymphocytes and offers the possibility of using this enzyme as a B lymphocyte marker.

## *Introduction*

In spite of their relatively homogeneous morphological appearance, lymphocytes can be divided into two major subpopulations, B and T, on the basis of certain immunologic characteristics. The development of a technique for separation of large number of B and T lymphocytes from normal blood [1] had made it possible for us to examine some of the bio-

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chemical characteristics of these cells. Of particular interest has been the activity of certain acid hydrolases such as acid phosphatase (ACP) and $\beta$-glucuronidase ($\beta$G) Using biochemical techniques the activities of these enzymes have been reported to diminish in the blood lymphocytes of patients with chronic lymphocytic leukemia [3 4 6 7 12, 14 16] a monoclonal proliferation most frequently composed of B lymphocytes [2, 11 15 23 26] Acid phosphatase and $\beta$-glucuronidase have also been used as cytochemical markers in distinguishing B from T lymphocytic malignancies [8–10 13 22, 27 28 30] In the present study we wished to determine whether the already demonstrated differences in activity of ACP and $\beta$G are in fact characteristics of the B or T nature of the normal lymphocytes, or whether they are due to an abnormality of the malignant lymphoid cells. For this purpose we prepared lymphocytes rich either in B or T cells on which biochemical determination of acid hydrolases was carried out. We hoped to demonstrate a biochemical difference between normal B and T lymphocytes that would be useful as a marker for classifying the lymphocytic cells into B or T category

## *Materials and Methods*

Leukocytes were obtained from 3 normal donors using the Aminco 'Celltrifuge' continuous flow blood cell separator (American Instrument Co., Silver Springs, Md.) [29] Platelets were removed as has been described elsewhere [5], while mononuclear cells were isolated using the Ficoll Hypaque technique [1]. After counting cells using a standard technique, approximately $2.5 \times 10^{9}$ cells were resuspended in RPMI 1640 medium, containing 5% agamma human serum (Biocell, Los Angeles, Calif.) at a concentration of $1.5–2.4 \times 10^{6}$/mL. In order to remove monocytes the cell suspension was poured into Falcon plastic petri dishes. The cells were allowed to stand for 2 h in a 5% $CO_2$ 95% air atmosphere at 37 °C. After gentle swirling, lymphocytes were pipetted off and their recovery was determined, and the cell number was adjusted $1 \times 10^{7}$ cells/ml for the purpose of the rosetting procedure.

*Preparation of B and T Lymphocyte Populations*

The procedure of ABDOU *et al.* [1] was employed. Cells were allowed to form spontaneous sheep red blood cell (SRBC) rosettes (E rosettes) by incubating aliquots of 2 ml cells, 2 ml 1% SRBC and 1 ml human AB serum (absorbed with SRBC) in a 15-ml plastic tube for 15 min at 37 °C. The suspension was centrifuged at 250 *g* for 10 min and placed in a refrigerator (4 °C) for 60 min. The cells were then resuspended gently and 4 ml of Ficoll Hypaque (0–10 °C) was underlayered. After centrifugation at 700 *g* for 35 min at 10 °C, the cells in the interface (upper layer of the gradient) and the cells in the pellet were collected separately Cells from the pellet were treated with 0.84% NH Cl for 5 min at 37 °C in order to lyze the SRBC. De-

terminations for E rosette-forming cells and surface immunoglobulins were carried out in both the cells from the interface layer and the pellet.

*Assays for B and T Lymphocytes*

*E rosette formation.* The technique of POTVIN *et al.* [25] was employed. A volume of 0.25 ml of $1.5 \times 10^6$ lymphocytes from the pellet were placed into 5 ml plastic tubes separately and were mixed with 0.2 ml 1% SRBC in RPMI media and 0.1 ml AB-positive human serum (Microbiological Associates, Bethesda, Md.). The mixture was incubated for 15 min in 5% $CO_2$ atmosphere at 37 °C and centrifuged for 5 min at 250 g. The tubes were placed in refrigerator for 18 h at 4 °C. After careful resuspension of the pellets the percent of E rosette-forming cells was determined.

*Surface immunoglobulins.* The cells were stained for surface immunoglobulin at 4 °C by the method of PERNIS *et al.* [24]. Polyvalent rabbit antihuman immunoglobulin labeled with fluorescein isothiocyanate (Cappel Laboratories, Downington, Pa.) was used. After staining, the cells were washed three times for 10 min at 4 °C in Hanks' medium and resuspended in buffered glycerin (50% in 0.01 M phosphate buffer solution pH 7.2). One drop of each suspension (interface layer and pellet preparation) was mounted on a glass slide and was immediately observed under Leitz Ortholux II fluorescent microscope. In all cases 200 cells were counted using X94 fluoride oil immersion objective, both under phase contrast and epi-illuminated ultraviolet light.

*Cytochemical studies.* Peroxidase, Sudan black (SBB) and α-naphthyl acetate esterase (α-NA esterase) reactions [19, 31], as well as May-Grünwald-Giemsa (MGG) stains, were performed on smears prepared on glass slides from the cells of the interface layer and the pellet, in order to determine the contamination of the lymphocyte populations with monocytes.

*Assays for acid hydrolases.* Assays for acid phosphatase, β-glucuronidase, β-galactosidase, β-hexosaminidase, α-arabinosidase, α-galactosidase, α-mannosidase, α-glucosidase and pH 4.0 and pH 5.0 β-glucosidase were carried out on the lymphocytes of both the interface layer and the pellet. Enzyme activities were determined using 4-methylumbelliferyl derivatives as substrate as previously described [5]. Activities for β-glucosidase were measured at pH 4.0 and 5.0 with whole cell suspension. Activities for β-galactosidase, β-hexosaminidase, α-arabinosidase, acid phosphatase, β-glucuronidase, α-mannosidase, and α-glucosidase were measured at pH 4.4 on cells sonicated for 15 sec [5]. Protein was estimated by the method of LOWRY *et al.* [18]. The acid hydrolase activities were expressed as milliunits/milligram of protein.

## *Results*

The E rosette-forming and immunofluorescent properties of lymphocytes of the interface layer and the pellet are summarized in table I. The cells of the interface layer consisted predominantly of B lymphocytes (79–90%) and the cells of the pellet of T lymphocytes (89–94.5%). The

chemical characteristics of these cells. Of particular interest has been the activity of certain acid hydrolases such as acid phosphatase (ACP) and β-glucuronidase (βG). Using biochemical techniques the activities of these enzymes have been reported to diminish in the blood lymphocytes of patients with chronic lymphocytic leukemia [3, 4, 6, 7, 12, 14, 16], a monoclonal proliferation most frequently composed of B lymphocytes [2, 11, 15, 23, 26]. Acid phosphatase and β-glucuronidase have also been used as cytochemical markers in distinguishing B from T lymphocytic malignancies [8–10, 13, 22, 27, 28, 30]. In the present study we wished to determine whether the already demonstrated differences in activity of ACP and βG are in fact characteristics of the B or T nature of the normal lymphocytes, or whether they are due to an abnormality of the malignant lymphoid cells. For this purpose we prepared lymphocytes rich either in B or T cells on which biochemical determination of acid hydrolases was carried out. We hoped to demonstrate a biochemical difference between normal B and T lymphocytes that would be useful as a marker for classifying the lymphocytic cells into B or T category.

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*Table I* B and T lymphocyte marker studies from 3 normal donors

| Donor No | Cell preparation | E rosettes binding % of cells | E rosettes non-binding % of cells | Immunofluorescence lymphocytes positive % | Immunofluorescence lymphocytes negative % | Immunofluorescence nonlymphocytes positive % | Immunofluorescence nonlymphocytes negative % |
|---|---|---|---|---|---|---|---|
| 1 | interface layer (B cell rich) | 9 | 91 | 82 | 10 | 4 | 4 |
| | pellet (T cell rich) | 89 | 11 | 6 | 94 | 0 | 0 |
| 2 | interface layer (B cell rich) | 10 | 90 | 79 | 11 | 5 | 5 |
| | pellet (T cell rich) | 94.5 | 5.5 | 8 | 92 | 0 | 0 |
| 3 | interface layer (B cell rich) | 10 | 90 | 90 | 6 | 0 | 4 |
| | pellet (T cell rich) | 94 | 6 | 3 | 97 | 0 | 0 |

*Table II* Acid hydrolases (mU/mg protein) in B and T lymphocytes of 3 normal donors

| Enzymes | B lymphocytes 1 | B lymphocytes 2 | B lymphocytes 3 | T lymphocytes 1 | T lymphocytes 2 | T lymphocytes 3 | B/T (mean ± 1 SE) |
|---|---|---|---|---|---|---|---|
| Acid phosphatase | 13.92 | 22.34 | 21 16 | 14.03 | 23 08 | 29.81 | 0.890 ± 0.090 |
| β-Glucuronidase | 0.982 | 1.010 | 0.800 | 1 418 | 1.251 | 0.843 | 0.816 ± 0.074 |
| α Mannosidase | 0.797 | 0.914 | 0.695 | 0.551 | 0.845 | 0.399 | 1 423 ± 0.191 |
| β-Galactosidase | 2.94 | 2.76 | 1 77 | 3 47 | 3.35 | 1 70 | 0.904 ± 0.069 |
| β-Hexosaminidase | 14 75 | 20.41 | 11 42 | 16.04 | 21 77 | 12.09 | 0.934 ± 0.007 |
| α-Arabinosidase | 0.594 | 0.794 | 0.387 | 0.787 | 0.878 | 0.424 | 0.857 ± 0.051 |
| α-Galactosidase | 0.342 | 0.561 | 0.453 | 0.443 | 0.563 | 0.475 | 0.907 ± 0.069 |
| α-Glucosidase | 0.701 | 0.613 | 0 474 | 0.699 | 0.646 | 0.395 | 1.051 ± 0 076 |
| β-Glucosidase pH 4 | 0.0179 | 0.02999 | 0.02265 | 0.0380 | 0.05313 | 0 05345 | 0.486 ± 0.041 |
| β-Glucosidase pH 5 | 0.0501 | 0 08516 | 0.05008 | 0.05060 | 0.07305 | 0.05052 | 1.049 ± 0 058 |

monocyte contamination of the B lymphocyte-rich preparation was low (2–11%) as determined by a differential count after MGG stain and peroxidase SBB and α-NA esterase reactions. Monocytes were practically absent from the T lymphocyte-rich preparation. Viability of the cells was as high as 95–100% as determined by trypan blue staining.

The activity of acid hydrolases including ACP and βG was found slightly lower in B lymphocytes than in T lymphocytes (table II). The most significant differences observed were related to the α-mannosidase and pH 4.0 β-glucosidase activity (table II). The α-mannosidase activity was higher in the B lymphocyte rich population, as compared with the T lymphocyte rich population, while the pH 4.0 β-glucosidase activity was found lower in the B lymphocyte-rich population than in the T lymphocyte-rich populations. In addition, pH 4.0 β-glucosidase activity was found to be significantly lower than the pH 5.0 β-glucosidase activity in the B lymphocyte population (table II).

## *Discussion*

The activity of most of the acid hydrolases studied was found to be approximately the same or slightly decreased in the B lymphocytes when compared with T lymphocytes. However, a significantly higher activity of α-mannosidase in the B lymphocytes as compared to the activity of T lymphocytes was observed. Whether this finding can be used as a biochemical marker for the B lymphocytic proliferations requires further investigation.

Acid phosphatase and β-glucuronidase activities were consistently lower in the B lymphocyte-rich population. These findings are in keeping on one hand with the reported cytochemical observations of slightly reduced ACP and β-glucuronidase activity in the normal B lymphocytes as compared with T lymphocytes of the same individual [19, 21, 30], while on the other hand are in disagreement with the results reported by MEUSERS *et al.* [20]. These authors found by biochemical means an increased ACP activity in the normal B lymphocytes and they suggest that ACP might be used as a marker for the B lymphocytes. The reason for this difference regarding the ACP activity in the B lymphocytes between our study and MEUSERS *et al.* [20] is not clear. It is possible, however, that this is due to a different contamination of the B lymphocytes with monocytes, which are rich in ACP [5].

The lack of a significant difference on the ACP and βG activity between B and T lymphocytes in the present study indicates that the reported cytochemical findings of an increased activity of ACP or βG in certain T lymphocytic malignancies [8–10, 13, 27, 28, 32] and a reduced ACP and/or βG activity in CLL [3, 4, 6, 7, 12, 16] and cells from lymph node

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monocyte contamination of the B lymphocyte-rich preparation was low (2–11%) as determined by a differential count after MGG stain and peroxidase, SBB and α-NA esterase reactions. Monocytes were practically absent from the T lymphocyte-rich preparation. Viability of the cells was as high as 95–100% as determined by trypan blue staining.

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In summary although the number of cases studied is small, the results of the present work revealed that there is a slightly decreased activity in the majority of the acid hydrolases in the B lymphocytes as compared to the T lymphocytes of normal individuals, with the exception of $\alpha$-mannosidase activity which was found significantly higher in the B lymphocytes.

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# Survival and Significance of PNH Erythrocytes a Scanning Study

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*Key Words.* Paroxysmal nocturnal haemoglobinuria · Scanning electron microscopy · Complement-sensitive erythrocytes

*Abstract.* The SEM study of PNH erythrocytes which survived incubations with decreasing dilutions (from 1:640 to 1:20) of fresh compatible serum in isotonic solution has indirectly evidenced that spherocytes and spherostomatocytes are the most sensitive population to the complement lytic action, swelling is an osmotic consequence of their membrane hypersusceptibility. In such functional abnormality the swollen red cells are closely followed by the cribrous and pitted erythrocytes. A relationship has been found between percentage of the swollen cells and frequency of PNH haemolytic bouts. these are most likely to occur when spherocytes and spherostomatocytes are above 40% of cell. On the contrary SEM evaluation of swollen erythrocytes does not allow to express any long-term prognosis about the severity and the course of PNH, owing to the constantly variable marrow production of abnormal erythrocytes.

Paroxysmal nocturnal haemoglobinuria (PNH) is due to the onset of a variable proportion of excessively complement-sensitive erythrocytes, whether such a lytic system is activated by the classic (antibody-mediated) or the alternate (antibody-independent activation of C3 proactivator) pathway. Abnormal PNH red cells share the property of binding 5–7 times more C3 than normal [11].

Within the abnormal PNH red cell pool, two populations have been recently distinguished [12] according to their different membrane com-

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plement susceptibility Depending on the amount of fresh serum required to induce antibody (anti I)-mediated haemolysis, the complement hyper sensitivity is intermediate in the so-called PNH II red cells elevated in the PNH III red cells In fact, although both abnormal populations bind the same increased amounts of C3 the complement hypersensitivity of PNH II red cells is 3–5 times greater than normal, while that of PNH III red cells is 10–20 times greater

The nature of the PNH membrane defect has not yet been ascertained exactly Almost constantly low red cell acetylcholinesterase activity levels do not constitute a sufficient finding Also extensive biochemical investigations have failed to detect significant abnormalities in protein and/or phospholipid red cell membrane contents.

Electron microscopic investigations have provided conflicting results. According to some authors [4 9] no difference has been found between normal and PNH red cell membranes. On the contrary in the studies of others [2, 10] PNH erythrocytes were shown to possess an unusually pit ted and patchy surface with several clefts.

Avoiding as much as possible membrane artefacts due to low ionic strength exposition in a variable percentage of PNH erythrocytes the above-mentioned membrane alterations were able to be confirmed [7] whereas other cells had quite normal appearance On the other hand recent scanning electron microscope (SEM) studies [6 8] reported the presence in PNH erythrocytes of (a) marked variations in cell size due to the high reticulocyte counts (b) numerous thalassaemia like target cells, with a prominent protuberance raising from their concavity and (c) pits and deep craters, on the surface of most deformed elements However no cor relation could be stated between frequency of altered erythrocytes and severity of the haemolytic disorder [6 8]

By means of the SEM the ideal tool for investigating cell surfaces, in the present study the problem of PNH erythrocytes has again been considered, in the attempt to evidence nature and degree of red cell alterations as well as the occurrence of a possible relationship between such deformations and severity of the haemolytic disorder Such problem may be tackled (a) by inducing PNH red cell haemolysis with mechanisms similar to those occurring *in vivo* (b) by testing different complement-sensitivity of red cells with progressively decreasing serum dilutions, and then (c) by evidencing with SEM the features of the cells which survive in any dilution. Such a procedure will allow the indirect identification of the different complement-sensitivity degrees of the abnormal PNH red cells

## *Materials and Methods*

### *Case Reports*

4 adult (28- to 43-year-old) PNH patients, equally distributed between both sexes, were studied after obtaining their informed consent. In each case the PNH diagnosis was stated on the basis of history and standard laboratory methods (positivity of acidified serum test, according to DACIE and LEWIS [3]; positivity of the sucrose lysis test, according to HARTMANN and JENKINS [5]; reduced red cell acetylcholinesterase levels, obtained from the reticulocyte-rich blood fraction; low neutrophil alkaline phosphatase scores, except for 1 case with normal values; decreased haptoglobin levels; increased amounts of plasma-free haemoglobin and indirect bilirubin; occasional or persistent urinary excretion of haemoglobin and iron). The time of diagnosis of the haemolytic disorder averaged from 1 / to 4 years.

In each case both Ham test and sucrose lysis tests were performed with fresh serum of the same compatible donor. The patient's serum was never used, in order to avoid both false-negatives in Ham's test, and false-positives in the sucrose test. Moreover for avoiding possible transfusion-caused morphology artefacts in PNH erythrocytes, the examined patients had not been receiving blood transfusions for at least 1 month before the SEM investigations. Their haemoglobin levels averaged from 7.4 to 13.3 g%.

4 apparently healthy volunteers (1 female and 3 males, 19- to 36-year-old) with normal haematologic indices served as controls.

### *Haemolysis Tests*

Both control and PNH erythrocytes were examined with SEM under basal conditions. Successively PNH red cells, obtained from freshly drawn citrate blood, were incubated at room temperature for 1 h with fresh compatible serum diluted in isotonic sucrose (British Drug Houses, Poole, England), at standard (1:20) as well as at higher (1:40, 1:80, 1:160, 1:320, 1:640) serum dilutions. Control erythrocytes were incubated only with the lowest (1:20 and 1:40) serum dilutions.

The sucrose lytic system was preferred to Ham test, although this latter has proved to be little more specific for PNH diagnosis, because in the sucrose test ( ) haemolysis is greater involving all the complement-sensitive cells; (b) nearly all compatible fresh sera are efficient, without showing the unsteady lytic activity observed in Ham's test under the same conditions; ( ) both the unspecific binding of antibodies to abnormal PNH erythrocytes and probably the efficiency of later complement components are enhanced; (d) rare false-positives are restricted to some cases of megaloblastic and immune haemolytic anaemia, but their incidence becomes quite negligible at low serum concentrations, as in the present investigation. Therefore, the sucrose test seems more suitable for accomplishing *in vitro* haemolysis-inducing occurrences which may arise in PNH patients.

At the end of the various incubations, corresponding haemolysis values were assessed spectrophotometrically by determining the supernatant fluid optical density at 540 nm. Such values were then expressed as percentage of complete (100%) haemolysis. Surviving erythrocytes were recovered after slight centrifugation, rinsed three times in Eagle solution at room temperature, and prepared for SEM.

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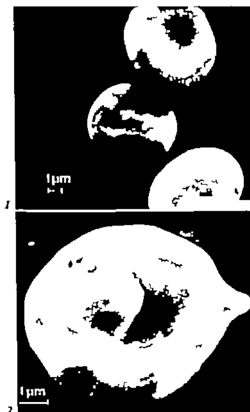



*Fig. 1.* PNH pitted erythrocyte retaining its normal morphology (top); cribrous element (center), typical spherocyte (bottom). × 4,800 10 kV 10° tilt.

*Fig. 2.* PNH pitted deformed erythrocyte × 15,100; 18 kV 10° tilt.

On the other hand, under the same basal conditions, SEM investigation of control erythrocytes evidenced only discocytes or very rare artefacts due to deficient cell fixation.

In order to detect an eventual relationship between SEM observed alterations and different complement-sensitivity degrees of PNH erythrocytes, the latter and control cells were incubated at room temperature for 1 h with decreasing fresh normal serum dilutions in isotonic sucrose.

In all investigated PNH patients, SEM study of lysis-surviving erythrocytes provided overlapping results: (1) higher than 1 640 serum dilu-

*SEM Methods*

The method suggested by BESSIS and WEED [1] was used two red cell fixation stages (1% glutaraldehyde for 30 min and then 0.5% osmium tetroxide in Eagle's solution at room temperature) were followed by progressive dehydration through a series of ethanol solutions of graded concentrations, up to 100%. Finally the red cells were dehydrated with propylene oxide.

After mounting on aluminium stubs, erythrocyte specimens were coated with a thin layer of gold-platinum, using a Galileo V7 vacuum chamber at $4 \times 10^{-5}$ Torr *at an incidence angle of 45 Erythrocytes were then examined with a Stereoscan* Cambridge Mark II A scanning electron microscope, at an accelerating voltage of 10–18 kV with 200 µm diameter illuminating apertures and 0.5 amp lens. Photomicrographs were recorded on Ilford FP4 125 ASA/35 mm films, at direct magnifications varying from 4 800 to 15 100 diameters.

## *Results*

The patients had not been receiving blood transfusions for 1 month before the SEM study Under such conditions the following conclusions may be drawn from the SEM studies (1) PNH erythrocytes present marked variations in cell size and shape the cell outline is often waved, crenated (2) a varying percentage of discocytes, averaging 32 8 ± 4.3% (mean ± SD) of cells, may be observed (3) a part of erythrocytes presents a pitted surface pits are distinct, sharp sufficiently spaced and are about 10–20 nm large some pitted cells retain normal morphology (fig 1 top) some show pronounced deformations, which consist mostly of marked protuberances arising from the peripheral outline, from the inner border (fig 2) or form the center of the hollow such deformations may appear single or associated The former group or unaltered pitted erythrocytes is less represented, averaging 7.3 ± 3.2% of cells. The latter group or deformed pitted erythrocytes is about two times more numerous, averaging 14 9 ± 5 6% of cells (4) a part of erythrocytes presents on their surface deep craters and depressions, as well as losses of membrane bits, so that a cribrous riddled-like aspect results (fig 1 center) such surface alterations are often associated with an initial or already advanced spherocytic deformation such cells, or cribrous erythrocytes average 18 7 ± 5 2% of cells (5) a varying fraction shows a marked spherocytic (fig 1 bottom) or spherostomatocytic deformation due to the marked increase in thickness on behalf of their diameter such erythrocytes appear similar to hereditary spherocytosis cells their incidence averages 25 8 ± 9 4% of cells.

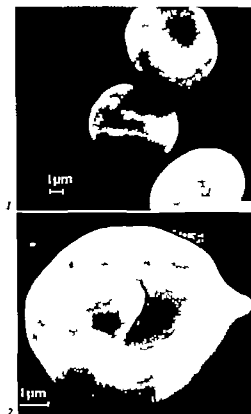



*Fig 1* PNH pitted erythrocyte returning its normal morphology (top); cribrous element (center) typical spherocyt (bottom) × 4,800; 10 kV 10° tilt.
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In all investigated PNH patients, SEM study of lysis-surviving erythrocytes provided overlapping results. (1) higher than 1 640 serum dilu-

tions failed to cause any variations in the above-mentioned red cell groups (2) a 1 640 dilution reduced the spherocytic population to about half (3) a 1 320 dilution resulted in the complete disappearance of both spherocytes and spherostomatocytes, as well as in a decrease (by about a fourth) of the cribrous group (4) a 1 160 dilution was followed by the disappearance of the cribrous cells and by a reduction (by about a third) of the deformed pitted erythrocytes (5) a 1 80 dilution resulted in the complete disappearance of both deformed and unaltered pitted erythrocytes.

Following lower (1 40 and 1 20) serum dilutions, the supernatant haemoglobin contents were found to persist unchanged while recovered cells appeared to be formed exclusively by discocytes. The complement hypersensitive fraction averaged from 68 to 77% of the PNH red cell pool Finally in each patient the spectrophotometrically evaluated percentage of the lowest dilution-resistant erythrocytes (from 23 to 32% of the cells) was found to agree significantly with the preincubation SEM assessed levels of discocytes.

SEM investigation of control erythrocytes incubated for 1 h at room temperature with the lowest (1 40 and 1 20) serum dilutions, failed to evidence any morphological abnormalities. Spectrophotometrically assessed haemolysis levels were constantly below 2.8% of cells.

## *Discussion*

Present SEM images of PNH red cells resulted quite similar to those previously reported [6 8] although the past studies had not paid too much attention to the presence of swollen cells in the PNH erythrocyte pool Likewise any relationship between percentage of PNH abnormal erythrocytes and severity of the haemolytic disorder was strictly refused [6 8] On the other hand, graded serum dilutions could demonstrate the existence within the abnormal red cell pool of two distinct populations ('PNH II red cells' and 'PNH III red cells') according to their different membrane complement susceptibility [12]

Incubation of PNH erythrocytes in progressively decreasing dilutions of fresh compatible serum in isotonic sucrose and successive SEM study of the recovered erythrocytes did indirectly show the most complement sensitive PNH population to be formed by spherostomatocytes and mostly by spherocytes. Even the slightest lysis inducing event will there

fore cause their disappearance. Thus the PNH III red cells must be morphologically identified with the spherocytes and spherostomatocytes.

Immediately lower serum dilutions evidenced that, in the lessening of the excessive membrane vulnerability to the complement, the swollen cells were closely followed by the cribrous erythrocytes. Such cells presented on their surfaces craters, depressions, and loses of membrane bits. Thus it may be deduced that the PNH II red cells have their morphological expression in the cribrous erythrocytes. In such groups the deformed' and 'unaltered pitted erythrocytes should be included as well, although their functional and morphological membrane abnormalities were shown to be less marked than in cibrous erythrocytes.

In sucrose lytic tests, however only a part of the cribrous and pitted erythrocytes was found to undergo lysis at the corresponding serum dilution, whereas the disappearance of the remainder occurred at the immediately lower dilution. Such functional differences in the membrane behaviour however only seem to be as such, since they must be accounted for not by a different complement susceptibility degree among cells of the same population, but rather by spatial causes. Only those C3 molecules, which are in close approximation to C42 sites, may in fact have a share in C5 cleavage [11]

In conclusion, in PNH patients only discocytes have shown to present a normal membrane resistance to the complement action. This statement has been confirmed by the finding that the proportions of the discocytes, assessed electron microscopically under basal conditions and spectrophotometrically after lysis of the most complement-sensitive red cells, were significantly overlapping. This means that, even at the lowest serum dilutions, discocytes undergo no lysis.

As far as the clinical usefulness of the present SEM results is concerned, unlike foregoing reports [6, 8] a relationship between proportion of swollen erythrocytes and tendency of the PNH patients to develop haemolytic bouts may be suggested. Although it cannot be assumed as a fast rule, in our experience a haemolytic access was most likely to occur if the level of the swollen cells was above 40%. The proportions of cribrous and pitted erythrocytes were generally shown to play a role of minor importance in inducing and maintaining haemoglobinuric exacerbations.

On the other hand, during the remission periods the levels of swollen erythrocytes were usually found to persist below 10–15 of cells.

Such findings would induce us to consider the complement lytic action as nearly constant its intensity is then modulated by the levels of circulat

ing swollen red cells and consequently by the variable marrow delivery of such elements. Of course this assumption does not exclude that sudden exacerbations of the complement activity may occur following immune reactions, infectious occurrences, and so on

In relation to the levels of the swollen red cells and, to a lesser degree, of the cribrous and pitted elements, SEM study of PNH erythrocytes allows to state only a short term prognosis about the impendence and probability of haemolytic bouts. No long-term prognosis may be reliably expressed owing to the well known constantly variable marrow production of abnormal erythrocytes

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# Enzymatic Abnormalities in Erythroleukemia[1]

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*Key Words* Enzymatic studies Erythroblasts Erythroleukemia Carbohydrate metabolism Esterases

*Abstract* Enzymatic studies were performed on erythroblasts obtained from marrows of 2 patients with untreated erythroleukemia Cytochemically erythroblasts showed abnormalities of several enzymes involved in carbohydrate metabolism, as well as abnormalities of specific and nonspecific esterases. Electrophoretic analysis of esterases extracted from predominantly erythroid marrows showed strong moderately fluoride resistant nonspecific esterase activity with α-naphthyl acetate, and weak activity with α-naphthyl butyrate. Isoenzymatic patterns of specific esterase activity in erythroleukemia were indistinguishable from those found in myeloblastic leukemia. The results are consistent with the concept of the DiGuglielmo syndrome in which a preleukemic erythroid disorder may precede the emergence of acute myeloblastic or myelomonocytic leukemia

The present report describes enzymatic studies of erythroblasts obtained from marrows of 2 patients with untreated erythroleukemia.

## Materials and Methods

Marrow was obtained by sternal puncture in a heparinized syringe from 2 patients with untreated erythroleukemia [1 6, 7 9 15] Films of marrow flecks were

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made between methanol-cleaned glass coverslips and stained with Wright' stain for conventional light microscopy Marrows were composed largely of erythroblasts (90% ) and most of the remaining cells were myeloblasts.

For cytochemical studies, separat marrow coverslips were stained for iron using the Prussian blue reagent [3], and glycogen using the periodic acid-Schiff (PAS) reagent with diastase digestion [9] Cytochemical tests for enzymes included phosphorylase [13], peroxidase [9], and specific and nonspecific esterase using combined staining technique [24]. Separate coverslips were also stained for nonspecific esterase in the presence of sodium fluoride [5, 24] Additional separate coverslips were stained for lactic dehydrogenase (LDH), malic dehydrogenase (MDH), succinic dehydrogenase (SDH), α-glycerophosphat dehydrogenase-S (α-GPD-S), α-glycophosphate dehydrogenase-M (α-GPD-M), glucose-6-phosphate dehydrogenase (G-6PD), and 6-phosphogluconic acid dehydrogenase (6-PGD) using the method of STUART and SIMPSON [21]. Reagents used in the latter method were obtained from Sigma Chemical Company St. Louis Marrow obtained from normal person served as control for each of these enzymes.

In the case of specific and nonspecific esterases, nonenzymatic analysis of esterases was also undertaken. Specific and nonspecific esterases were liberated from saline washed particles by ultrasonic disruption in cetyltrimethylammonium bromide [16]. Esterases were also extracted from separat marrow samples with the use of lysolecithin (Sigma, St. Louis, Mo.) according to established techniques [23] Marrow esterases contained in these extracts were subjected to electrophoresis in polyacrylamide gel and stained for specific and nonspecific esterases [16] Separate electrophoretic gels were stained for nonspecific esterase in the presence of sodium fluoride [5, 16]

## *Results*

As shown in figure 1 Wright-stained films of marrow demonstrated largely proerythroblasts (60%) and megaloblastoid intermediate macronormoblasts (approximately 30%). Many erythroblasts were gigantic and bizarre-appearing with multiple nuclei. Some erythroid precursors had abundant cytoplasm with multiple vacuoles, perhaps indicating that these were degenerating cells. Most of the remaining cells were myeloblasts.

Results of cytochemical studies are summarized in table I As found frequently in cases of erythroleukemia [8, 15 18] Prussian blue stain demonstrated numerous ringed sideroblasts, and the PAS stain for glycogen was intensely positive in the majority of the erythroid precursors. Coarse-appearing PAS positivity was observed frequently (fig. 2a). Diffuse PAS positivity was seen particularly in late megaloblastoid intermediate macronormoblasts. PAS positivity disappeared after diastase digestion.

Phosphorylase activity was intense in erythroid precursors at all stages of maturation, especially in giant multinucleate erythroblasts (fig. 2b). In

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# Enzymatic Abnormalities in Erythroleukemia[1]

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*Key Words* Enzymatic studies Erythroblasts Erythroleukemia Carbohydrate metabolism Esterases

*Abstract* Enzymatic studies were performed on erythroblasts obtained from marrows of 2 patients with untreated erythroleukemia. Cytochemically erythroblasts showed abnormalities of several enzymes involved in carbohydrate metabolism, as well as abnormalities of specific and nonspecific esterases. Electrophoretic analysis of esterases extracted from predominantly erythroid marrows showed strong moderately fluoride resistant nonspecific esterase activity with α-naphthyl acetate, and weak activity with α-naphthyl butyrate. Isoenzymatic patterns of specific esterase activity in erythroleukemia were indistinguishable from those found in myeloblastic leukemia. The results are consistent with the concept of the D Guglielmo syndrome in which a preleukemic erythroid disorder may precede the emergence of acute myeloblastic or myelomonocytic leukemia

The present report describes enzymatic studies of erythroblasts obtained from marrows of 2 patients with untreated erythroleukemia

## *Materials and Methods*

Marrow was obtained by sternal puncture in a heparinized syringe from patients with untreated erythroleukemia [1 6, 7 9 15] Films of marrow flecks were

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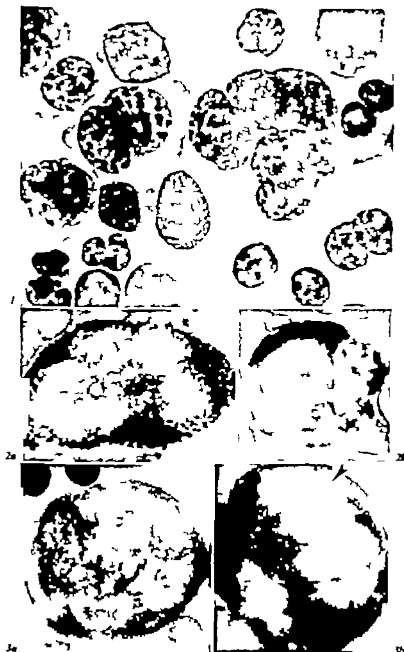

1
2a
2b
3a
3b

*Table 1* Cytochemical abnormalities of marrow erythroblasts in erythroleukemia

| | |
|---|---|
| Prussian blue (iron) | ringed sideroblasts |
| PAS | 4 + |
| Phosphorylase | 4 + |
| Nonspecific esterase | 4 + (diffuse staining, incompletely inhibited by sodium fluoride) |
| Specific esterase | 3 + |
| Peroxidase | − 3 + |
| LDH | 4 + |
| MDH | 3 + |
| SDH | 0 |
| α-GPD-M | 0 − 1 + |
| α-GPD-S | 1 + |
| G-6PD | 2 + |
| 6-PGD | 0 |

4 + = Coarse abundant granularity in the majority of erythroid precursors 3 + = punctate granularity in most erythroid precursors ± + = occasional granules in some erythroid precursors 1 + = rare granules in some erythroid precursors 0 = no granules visible.

tense nonspecific esterase activity was observed in most of the erythroblasts [20] and was especially conspicuous in large multinucleate cells (fig 3a) Using α-naphthyl acetate or α-naphthyl butyrate the nonspecific esterase activity in erythroblasts was intense, and incompletely inhibited by sodium fluoride. Specific esterase activity was also detectable in most of the erythroid precursors at all stages of maturation (fig 3a) and in

*Fig 1* Erythroleukemia, bone marrow showing giant multinucleate erythroblasts, megaloblastoid intermediate macronormoblasts and several myeloblasts. Wright s stain. × 1,500.

*Fig 2* *a* Giant multinucleate erythroblast stained with PAS reagent. Large coarse-appearing deposits of glycogen are seen in the cytoplasm. × 1,500. *b* Binucleate erythroblast showing intense cytoplasmic phosphorylase activity (black). × 1,500

*Fig 3* *a* Giant multinucleat erythroblast stained for both nonspecific esterase (α-naphthyl acetate) nd specific esterase (naphthol ASD-chloroacetate). The dark gray cytoplasmic coloration represents intense nonspecific esterase activity that appears bright crimson unde the light microscope. Black punctate granules scattered throughout the cytoplasm indicate specific esterase activity × 1,500. *b* Giant mul tinucleate erythroblast stained for peroxidase using o-tolidine. The arrow points to a cluster of peroxidase-positi e granules in the cytoplasm. × 1,500

myeloblasts. Clusters of peroxidase-positive granules could be observed in many of the erythroblasts, particularly the large multinucleate cells (fig. 3b) and in myeloblasts.

Of the various enzymes demonstrable by the formazan technique, lactic dehydrogenase showed the highest activity in erythroid precursors (fig. 4). Malic dehydrogenase activity was also strong, and activity of G-6PD was moderate. Scant activity of -GPD-S was observed. Activity of succinic dehydrogenase, α-GPD-M, and 6-PGD could not be detected in erythroblasts.

In coverslips of marrow obtained from a normal person, none of the enzymes or other substances demonstrable in the erythroleukemia erythroblasts could be detected in normal erythroid precursors.

Electrophoretic patterns of specific and nonspecific esterases obtained from *predominantly erythroid marrows* are illustrated in figure 5. In the gel stained for specific esterase using naphthol ASD-chloracetate, five bands could be seen. In the gel containing esterases extracted with cetyltrimethylammonium bromide and stained with α-naphthyl acetate for nonspecific esterase, two bands were demonstrable. In the gel containing esterase extracted with lysolecitin, four nonspecific esterase bands were observed when α-naphthyl acetate was used as the substrate. When α-naphthyl butyrate was used as the substrate, one weakly stained band was visualized in the cetyltrimethylammonium bromide extract, and three bands were seen in the lysolecithin extract. Addition of sodium fluoride to the incubation mixture incompletely inhibited nonspecific esterase in all instances and in some gels, particularly those stained with α-naphthyl acetate, activity was inhibited but not obliterated.

## *Discussion*

In the present studies, several enzymatic abnormalities were noted in erythroleukemia erythroblasts. Markedly increased activities of lactic and malic dehydrogenases as well as increased acti ity of G-6PD in erythroblasts suggest that there may be abnormalities of both Embden-Meyerhof and pentose-phosphate shunt pathways of carbohydrate metabolism in

stained with α-naphthyl acetate. *D* Esterase extracted with cetyltrimethylammonium bromide and stained with α-naphthyl butyrate to demonstrate nonspecific esterase activity. *E* Esterase extracted with lysolecithin and stained with α-naphthyl butyrate.

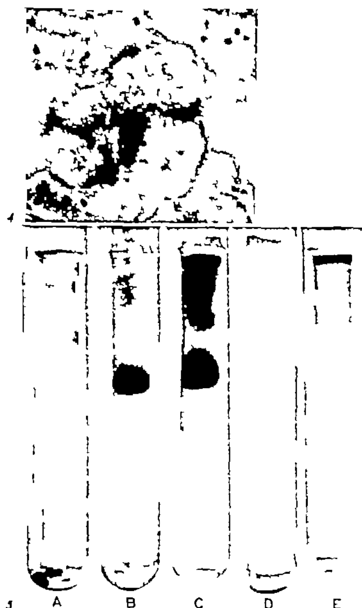



*Fig 4* Intense lactic dehydrogenase activity (black formazan granules and dif fuse bla k deposits) in a giant multinucleate erythroblast × 1,500

*Fig 5* Polyacrylamide disc electrophoretic p tterns of specific and nonspecific esterases extracted from the marrow of patient with erythroleukemia. *A* Esterase extracted with cetyltrimethylammonium bromide and stained with naphthol ASD-chloroacetate to demonstrate specific esterase acti ity *B* E terase extra ted with cetyltrimethylammonium bromide and sta ned w th α-naphthyl acetate t demonstrate nonspecific esterase cti ity *C* E terase extracted with lysolecithin nd

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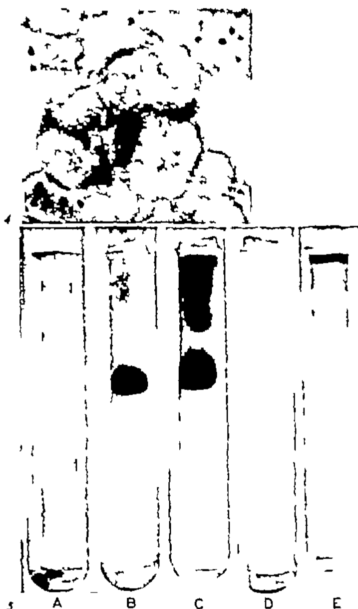



*Fig 4* Intense lactic dehydrogenase activity (black formazan granules and diffuse black deposits) in a giant multinucleate erythrobla t × 1,500.

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erythroleukemia. Further cytochemical evidence for abnormalities of carbohydrate metabolism may be reflected by unusually increased phosphorylase activity As in other abnormal erythroblasts that contain abundant glycogen [10 11] increased phosphorylase activity in erythroleukemia may reflect reversibility of the glycogen degradation reaction favoring biosynthesis and deposition of glycogen [2 22]

Using α-naphthyl acetate or α-naphthyl butyrate nonspecific esterase activity found in monocytes is particularly sensitive to inhibition by fluoride, whereas nonspecific esterase in cells of granulocytic origin is relatively fluoride resistant [10 24] In erythroleukemia, nonspecific esterases were incompletely inhibited by sodium fluoride as are granulocytic type nonspecific esterases Electrophoretically nonspecific esterases extracted from predominantly erythroid marrows in erythroleukemia showed a different electrophoretic pattern than those in myeloblastic leukemia [14] These observations suggest that isoenzymatically erythroleukemia nonspecific esterases differ from those in myeloblastic leukemia although both show a similar resistance to inhibition by sodium fluoride

Specific esterase is a marker for cells of granulocytic origin [24] Erythroblasts from patients with acute erythremic myelosis [10] and erythroleukemia [12] contain specific esterase activity suggesting that these erythroblasts may have a common myeloblastic stem cell as demonstrated cytogenetically in chronic erythroleukemia [17] and chronic granulocytic leukemia [19] On isoenzymatic analysis, specific esterases extracted from predominantly erythroid marrows in erythroleukemia show electrophoretic patterns indistinguishable from those in myeloblastic leukemia [14] Accordingly the results obtained are consistent with the viewpoint that erythroleukemia is part of the framework of the DiGuglielmo syndrome [4 14] in which the final common pathway is sometimes acute myeloblastic or myelomonocytic leukemia

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*Case Report*

A 56-year-old female was first admitted in March 1971 with generalized weakness and carpal spasms. There w no family history of any note. From 1945 she has been troubled occasionally by loose stools several times day 2 weeks prior to her admission, she developed severe diarrhea and on admission was weak, pale and cachectic. Physical examination revealed clubbing of the fingers and carpopedal spasms. There was no lymphadenopathy or splenomegaly I the course of lengthy hospitalization and detailed investigations, malabsorption syndrome was revealed probably secondary to acquired agammaglobulinemia Hemoglobin was 9 g/100 ml, reticulocytes 1.7%, and bilirubin 0 9 mg 100 ml Immunoglobulin determination showed IgG 50 mg/100 ml and no detectabl IgM or IgA The Coombs' test was negative. The bone marrow aspirate showed total lack of plasma cells in addition to an absence of iron granules and number of giant bands Following treatment with folic acid and iron, the hemoglobin level rose t 10 g 100 ml

The patient was given 6.0 g of SZN daily All the objective and subjective signs of malabsorption regressed and the patient was discharged on daily SZN treatment The patient was well until September 1972 when weakness, unassociated with diarrhea, recurred On admission she was pale without new physical findings. Hemoglobin level was 6.3 g/100 ml, reticulocytes 60%/ and methyl violet staining revealed typical Heinz bodies in most of the erythrocytes. The leukocyte count and differential were normal. Plat let count, total and direct reacting bilirubin, and haptoglobin levels were normal Direct and indirect Coombs tests were negative. All three major immunoglobulins were totally absent from the serum. The bone marrow showed prominent hyperplasia of the erythroid series with mild dyserythropoietic changes. Again plasma cells were not seen The activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase and glutathione peroxidase was high. The glutathione level and its stability ere normal and no pathological or heat-labile hemoglobin as found Osmotic fragility and autohemolysis were markedly increased

Heinz body hemolytic anemi was diagnosed nd the SZN therapy was stopped. The hemoglobin level gradually rose ith parallel reduction in the number of reticulocytes. Within 3 days of cessation of treatment, the Heinz bodies disappeared from the red cell On discharge, the patient hemoglobin was 11 7 g 100 ml with 3.8% reticulocytes, and returned to normal levels 2 weeks later All the enzymatic, osmotic, and hemolytic abnormalities described above disappeared

*Discussion*

Hemolytic anemia associated with Heinz bodies in the erythrocytes may be caused by the presence of a pathological, unstable hemoglobin which undergoes denaturation and precipitation [3 11] or when red cells are submitted to an oxidati e stress The latter situation is particularly common in erythrocytes deficient in one of the enzymes associated with

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# Salicylazosulphapyridine-Induced Heinz Body Anemia

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Tel Hashomer and Tel Aviv University Medical School, Tel Aviv

*Key Words* Salicyclazosulphapyridine Hemolytic anemia Heinz body anemia Malabsorption Agammaglobulinemia

*Abstract* A 58-year-old patient suffered from severe malabsorption due to agammaglobulinemia. Treated empirically with salicylazosulfapyridine 2–6 g/day the subjective and objective features of malabsorption regressed. About a year after this treatment she developed a generalized weakness without renewal of the diar rhea the diagnosis of Heinz body hemolytic anemia was established In our patient, hemolysis began many months after the commencement of treatment and no deficiency of G6PD or other erythrocyte enzyme or pathological hemoglobin were found

## *Introduction*

Salicylazosulfapyridine (SZN) has been widely used in the treatment of ulcerative colitis since 1942. There is no proof that the drug changes the microflora of the intestine in any way and its mode of action is, in fact not known [14] The drug is effective in at least a proportion of pa tients with ulcerative colitis. The side effects described include nausea, vomiting skin rash fever joint pains, agranulocytosis [22] pancreatitis [4] and yellow pigmentation of the skin [24] Only a few cases of hemolysis have been described [5–7 9–12 18 20 22] In these cases, the ane mia appeared soon after the commencement of treatment and in those pa tients examined a deficiency of red cell G6PD was found

We present a patient who developed a hemolytic anemia with the appearance of Heinz bodies in the erythrocytes about 14 months after the institution of treatment with SZN

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Heinz body hemolytic anemia was diagnosed and the SZN therapy was stopped. The hemoglobin level gradually rose with parallel reduction in the number of reticulocytes. Within 3 days of cessation of treatment, the Heinz bodies disappeared from the red cells. On discharge, the patient's hemoglobin was 11 7 g/100 ml with 3.8% reticulocytes, and returned to normal levels 2 weeks later. All the enzymatic, osmotic, and hemolytic abnormalities described abov disappeared

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## Discussion

Hemolytic anemia associated with Heinz bodies in the erythrocytes may be caused by the presence of a pathological, unstable hemoglobin which undergoes denaturation and precipitation [3, 11] or when red cells are submitted to an oxidative stress. The latter situation is particularly common in erythrocytes deficient in one of the enzymes associated with

the hexosemonophosphate pathway particularly glucose-6 phosphate de hydrogenase, when they come into contact with certain agents. These agents such as the antimalarials and sulphonamides, react with oxyhemoglobin in the red cell and produce hydrogen peroxide [8] In normal erythrocytes the peroxide radicals are broken down by glutathione peroxidase which is coupled with the glutathione and hexosemonophosphate pathways In situations where this system is defective a as result of genetic deficiency as well as in situations where the oxidative stresss overwhelms the neutralizing ability of the red cell the peroxide radicals act on the hemoglobin and bring about the production of Heinz bodies which are precipitated and denatured hemoglobin aggregates [17] These precipitates usually become attached to the sulfhydryl groups of the cell membrane and by an unknown mechanism possibly by causing rigidity of the cell membrane and impairment of its deformability cause their trapping in the reticuloendothelial system and removal from the circulation [16, 21 23]

SZN is a sulfa derivative and a number of cases in which this drug gave rise to hemolytic anemia have been described In 1958 SPRIGGS and OXON [22] reported 3 cases of Heinz body hemolytic anemia in patients with ulcerative colitis who were treated with SZN The anemia and the Heinz bodies disappeared on cessation of treatment [22] Red cell G6PD levels were not studied in these patients In the same year KELLERMAYER *et al* [18] described hemolysis of erythrocytes deficient in G6PD by SZN amongst other agents. In 1968 COHEN *et al* [9] described 2 cases of hemolytic anemia associated with SZN and in both patients a G6PD deficiency was demonstrated. Recently DAS *et al* [12] described the adverse reactions during SZN therapy in 133 patients. 5 patients had evidence of hemolytic anemia 2 had Heinz bodies present. The dose varied from 3 to 8 g/day and the hemolysis was noted after 2–3 weeks of therapy All 5 patients were slow acetylator phenotypes, but no G6PD estimations were carried out Our patient differs from most cases described in the literature in that the drug caused a Heinz body hemolytic anemia in the absence of a pathological hemoglobin or enzymatic deficiency A similar case where sulfadiazine caused Heinz body anemia has recently been published [1] It is reasonable to assume that the hemolysis was the result of overloading and overwhelming of a normal enzymatic system by the drug The second unusual aspect in our patient is the late onset of the anemia in contrast to most reported cases where it appeared several weeks after therapy was started. One can only speculate that some unknown oxidative insult as a

latent infection, triggered the acute hemolytic episode It is also possible that a compensated hemolytic process antedated the patient s admission.

SZN is given as a treatment for various intestinal disorders, particularly ulcerative colitis and Crohn s disease In the patient described, SZN was given as a treatment of malabsorption complicating agammaglobulinemia [2, 13 15] Anemia is often a feature of the mentioned diseases as a result of deficiency of iron, folic acid or vitamin B $_{12}$. Hemolysis may also be a feature of the disease itself [19] It is therefore important to emphasize that those patients treated with SZN may develop anemia also as a result of the treatment, and it is advisable to perform regular blood counts in these patients.

*Acknowledgement*

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## Short Communication

Acta haemat. 59: 315–316 (1978)

# Hyperimmune Thrombocytopenia in Pregnancy Treated by Splenectomy

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*Key Words.* Hyperimmune · Thrombocytopenia · Pregnancy · Splenectomy

*Abstract.* 3 cases of hyperimmune thrombocytopenia in pregnancy are described. Splenectomy was followed by return of the platelet count to normal in all. There was no maternal or fetal death.

Hyperimmune thrombocytopenia (HTP) in pregnancy represents a hazard to both mother and fetus. Prednisone is the treatment of choice but may be ineffective when HTP occurs in pregnancy. Splenectomy offers an early alternative in severe cases.

### *Case Reports*

*Case I*

A 20-year-old woman who had previous normal pregnancy presented at 20 weeks gestation with severe epistaxis and purpura after rubella exposure. Haemoglobin was 9.5 g/dl and the platelet count was $4 \times 10^9$/l. Increased immature megakaryocytes were seen on marrow aspiration. Rubella HAI titre was 320 and subsequently fell to 10. Bleeding continued unresponsive to transfusion of 12 U of platelet concentrate and splenectomy was performed. Bleeding ceased thereafter and the platelet count returned to normal in 10 days. A full-term infant who did not develop thrombocytopenia was later delivered.

*Case II*

A 37-year-old woman with eight previous pregnancies presented at 36 weeks gestation. Her platelet count was $12 \times 10^9$/l, haemoglobin 12 g/dl, and she had wide

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Acta haemat. 59: 317–318 (1978)

# Therapeutic Response of Blastic Crisis of Chronic Myeloid Leukemia (CML)

G. Castrola, G. Ippoliti, R. Invernizzi and G. Marini

To the Editor

It has been observed for some time that in the course of blastic crises of CML, two types with different reactivity to therapy [1] can be distinguished, one being cytochemically lymphoid. The constant presence of the Ph1 chromosome, even in the lymphoid type, suggested that it was due to profound enzymatic and metabolic alterations of the granulocytic line [1, 2]. Nevertheless, recent experimental data lead some authors to accept their lymphatic nature [3, 4].

In the past 3 years, we observed 20 patients who, according to generally accepted criteria [2], suffered from blastic crisis of CML. Chromosomal analysis was performed both on peripheral blood and bone marrow by means of the Q-banding technique. 17 cases, where mitoses could be analyzed, clearly show the constant presence of the Ph1 chromosome: one case revealed double and triple Ph1 chromosome. 4 cases out of 20 presented cytochemical characteristics of the lymphoid type and were treated by the therapy proposed by Canellos *et al.* [5]: 3 of them reached complete remission for 6 months after treatment with VCR and prednisone. Of the 16 nonlymphatic type cases, only 2 reached complete remission with daunorubicin, cytosine arabinoside and 6-thioguanine, whereas 4 reached only partial remission and 10 were insensitive to the therapy employed. The difference in response to therapy, together with the particular sensitivity to antilymphoblastic therapy of the first type, encourage us to believe that a classification of the cytological type may furnish helpful indications of the most effective therapeutic approach.

spread purpura. Antibody screen was negative. A marrow aspirate showed increased immature megakaryocytes. Despite prednisone 60 mg/day her platelets fell to $6 \times 10^9$/l, accompanied by severe epistaxis over the next 5 days. The splenic pedicle was clamped with peroperative platelet transfusion and a 3000 g infant was delivered by Caesarian section. A splenectomy was then performed. Cord blood platelets were $11 \times 10^9$/l. The baby's count was $95 \times 10^9$/l, which fell to $30 \times 10^9$/l on the 3rd day after delivery but returned to normal 2 days later. The mother's platelets were normal 7 days post-operatively and have remained so since.

*Case III*

A 30-year-old woman who had two previous pregnancies presented with purpura of her lower limbs at 20 weeks gestation and a history of rubella exposure. Her platelet count was $66 \times 10^9$/l, and a marrow aspirate showed increased immature megakaryocytes. Therapy with prednisone 80 mg/day was initiated. Her platelet count steadily decreased and she presented 3 weeks later with severe purpura, epistaxis and gingival bleeding, her platelet count being $1 \times 10^9$/l. A splenectomy was performed under platelet cover and her count returned to normal over 2 months. She gave birth to twins at term who had stigmata of congenital rubella.

## *Discussion*

HTP may present or worsen in pregnancy. It is a rare complication and reports are anecdotal. Corticosteroids alone rarely achieve remission [1] and represent a small theoretical hazard to the fetus [2]. As splenectomy has become safer with the availability of platelet transfusions, it has become an early alternative in severe thrombocytopenia.

Thrombocytopenia results in a high fetal and neonatal loss although maternal mortality is low [3]. Elective Caesarian section may minimize fetal trauma and in extreme circumstances may be combined with splenectomy as described.

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N J Bryant An Introduction to Im                ogy Saunders, London 1976. XXI + 255 pp., £ 9 75 ISBN 0-7216-2170-8.

I the first part of this book, the theoretical bases of immunohaematology is presented in concise way A short but clear account of genetics and general immunology is followed by more detailed discussion f the various blood group systems. A very brief section is devoted to the haemolytic diseases. In the second practical part methodology of donation of blood, production of blood components, storage of blood and blood grouping reagents and the use of various materials in blood transfusion is outlined. A large section on techniques follows with much valuable practical advice.

The theoretical part includes great number of informative illustrations. However some f these seem unnecessarily space-consuming.

Each small chapter is followed by 'typical examination questions Although these questions are specifically designed for candidates of the 'Canadian Society of Laboratory Technologists they will certainly be usefull to many other students of this field. The book ends with an extensive glossary and an exhaustive list of ed cell antigens. It may be regretted that the HLA system is not included or not even mentioned At least this would have been necessary in connection with the chapter on platelet transfusions. Or else the title of the book should make it clear that it is restricted to red cell immunology

On the whole this is very useful ntroductory text which will certainly serve not only students of medical technology as is modestly understated in the uthor' preface, but also medical students and physicians interested in haematology and immunology

U Bucher, *Bern*

R. E. Cotton (ed) Histopathology Official Journal of the British Division of the International Academy of Pathology Blackwell, Oxford.

*Histopathology* new journal which will be published bi-monthly is designed to be forum for pathologists who practize diagnostic and/or investigative histopathology and thereby contribute t the better understanding of disease in man. If the January 1977 issue (vol. 1, N 1) in quality of both content and printing is representative for the future format of the journal, editorial enthusiasm about this publication will be shared by the subscribers. The members of the Boards of Editors and Editorial Advisors represent persuasive assurance that *Histopathology* meets the promise 'to be different' and becomes significant journal in its field.

M. W Hess, *Bern*

B Dupont and R. A Good Immunobiology of Bone Marrow Transplantation. Grune & Stratton, New York 1976 XVI+346 pp., US$ 28.75 ISBN 0-8089-0982 7

*Immunobiology of Bone Marrow Transplantation* is the hardcover edition of the September and December 76 issues ( ol. VIII/3 and 4) of the Transplantation Pro-

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# Double Test of Spontaneous Rosettes with Sheep and Mouse Erythrocytes

## Statistical Studies and Usefulness in Malignant Evolutive and Nonevolutive Lymphoproliferative Diseases

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*Key Words.* Rosette forming cells · Lymphoproliferative diseases · Chronic lymphoid leukemia · Lymphocytic well differentiated lymphosarcoma

*Abstract.* We have demonstrated the log normality of the distribution of sheep rosette-forming cells and mouse rosette forming cells' values obtained with lymphocytes isolated from the peripheral blood of 135 healthy human beings and 57 patients suffering from chronic lymphocytic leukemia and well differentiated lymphocytic lymphosarcoma with bone marrow infiltration either in evolution or in remission. In evolutive cases, the absolute numbers of mouse rosette-forming cells rose as well as the lymphocytosis, whereas the absolute numbers of sheep rosette-forming cells were normal or even increased in spite of an impressive drop of their percentage. In nonevolutive cases, the absolute numbers of sheep rosette-forming cells and mouse rosette-forming cells were lowered to half of the normal values as consequence of the lymphopenia induced by chemotherapeutic agents.

By using immunological surface markers, it is possible to identify different lymphoid subpopulations in the blood of normal human beings and in patients suffering from lymphoproliferative diseases. The T lymphocytes, thymus dependent cells, are able to form rosettes with Sheep red blood cells (SRBC) [4, 12, 28]; the B lymphocytes, bursa equivalent derived, have specific surface markers [2]: membrane-bound surface immunoglobulins [24] and receptors for the Epstein-Barr virus [13].

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ceedings. Presentations at the first International Bone Marrow Transplantation Conference of November 1975 at Memorial Sloan Kettering Cancer Center New York, are summarized in a comprehensive manner A large part of the book is dedicated to *graft versus-host-reaction (GVHR) one of the central problems in bone marrow* transplantation. Biological factors controlling GVHR, the relative importance of histocompatibility determinants as well as different *in vitro* models for the generation of GVH reactive lymphocytes are discussed by several authors. One paper summarizes the pathology of GVHR In a second part, results are presented on several experimental animal models investigating problems such as the phenomenon of genetic resistance to hemopoietic stem cell engraftment, the influence of preceeding blood transfusions upon graft rejection, autologous bone marrow reconstitution after ALG conditioning for semicompatible marrow transplantation and graft versus-leukemia effects. Finally in a third part, clinical application of bone marrow transplantation (bone marrow aplasia, leukemia, severe combined immunodeficiency) including several papers on immunologic reconstitution after bone marrow transplantation as well as diagnosis and treatment of infection complication, are presented All articles are accompanied by a reference list The book is an excellent help to hematologists and clinicians working in the field of human bone marrow transplantation.

J GMÜR

J F DAVIDSON, M M SAMAMA and P C. DESNOYERS (ed) **Progress in Chemical Fibrinolysis and Thrombolysis**, vol 2. Methodology Raven Press, New York 1976 212 pp. US$ 16.50. ISBN 0-89004-136-9

This second volume is exclusi ly ded cated to analyt cal procedures in fibrinolysis with some closely related assay methods, the estimation of soluble fibrin monomer the ethanol gelation and the protamine sulfate tests involved in the detection of disseminated intravascular coagulation which frequently precedes a fibrinolytic state *in vivo* The classical methods euglobulin lysis time the fibrin plate, the assay of plasminogen and inhibitors, the assay of essel wall acti ity and the fibrin slide method ha e all been considered Unfortunately one does not find any description of quantitative assay methods for the activators urokinase and streptokinase.

These methods are well described, and the difficulties and pitfall commented *upon which makes their use directly possible without further information. The 22* uthors (strangely enough 2 of these have been forgotten in the list of contributors) give the methods they use in their own laboratory However each chapter i complet ed by references. This book of f brinolytic methodology is very useful for the laboratory

F DUCKERT *Bas l*

phocyte suspension (4·10⁶ cells/ml) was mixed to SRBC or MRBC in equal volumes in HBSS added up with 12.5% of fetal calf serum (Gibco Bio-Cult, Paisley Scotland) using ratio erythrocytes/white cells = 30 [4]. Two incubations, the first at 37 °C for 10 min and the second at 4 °C for 1.30–6 h were separated by centrifugation *at 200 g for 10 min. After gentle resuspension, the rosettes, defined as lym*phocytes which bound three or more heterologous erythrocytes, were enumerated in duplicate samples in Burker's chamber. For each sample, at least 800 leukocytes were counted. The control tubes, with RBC alone, never contained any clusters.

The lymphocyte percentage in the cell suspension was evaluated after counting 200 cells on May-Grünwald-Giemsa stained smears. In the normal subjects, the percentage of lymphocytes reached 95.1 % ± 0.3, whereas in the patients entering remission after chemotherapy the isolated lymphocytes were contaminated not only by monocytes as in the control subjects, but also by immature myeloid cells. The results of RFC were expressed as percentage of the lymphocytes isolated and in *absolute number per cubic millimeter of blood. A SRFC/SRFC + MRFC ratio* was established for each subject.

By cumulative distribution of the studied parameters on a probit scale, it was possible to determine whether the two pathological populations obeyed logarithmo-normal law as in the control subjects. Moreover we compared the geometric means of the different groups (controls, patients in clinical progression, and patients in remission) by the Student's t test. The immune-adherence properties of several patients were also regularly followed up and their modification compared with the variations of the blood lymphocytosis.

## *Results*

In all the groups of normal (group A) and sick (groups B and C) subjects, the percentage and absolute number of the two types of rosettes as well as the SRFC/SRFC + MRFC ratio were distributed along a logarithmo-normal curve.

Figures 1 and 2 which represent the individual data obtained for the absolute numbers of SRFC and MRFC clearly show that the cases of *LLS* had the same distribution as those of *CLL*.

In the patients with evolutive CLL and LLS (group B), the SRFC proportion was decreased, whereas the percentages and absolute numbers of lymphocytes and MRFC were highly increased (table I II). Nevertheless, the absolute number of SRFC was either normal or moderately high. In all cases, the values of the SRFC/SRFC + MRFC ratio were diminished (table I, II).

5 of our patients had different RFC patterns in spite of obvious manifestations of lymphoproliferative disease (table III) 4 of them, who had a reduction in the SRFC percentage with either normal or low absolute

It is known that in chronic lymphocytic leukemia, the percentage of T cells forming spontaneous rosettes with sheep erythrocytes is decreased [9 12, 23 27 29] on the other hand, a high proportion of lymphoid cells form rosettes with mouse erythrocytes [25] According to Stathopoulos and Elliott [25] the mouse rosette forming cells (MRFC) which are less frequent in normal subjects than in leukemic patients, probably represent B cells.

In a previous paper [5] we reported that the modifications of the immune-adherence properties – sheep rosette-forming cells (SRFC) and MRFC – were not only observed in cases of chronic lymphocytic leukemia (CLL) but also in patients with malignant lymphomas of the well-differentiated lymphocytic type in stage IV Our present observations complete this preliminary work by a mathematical approach of the immunological status using SRFC and MRFC in patients with CLL and lymphocytic lymphosarcoma (LLS) of this particular type. In several patients, the influence of chemotherapy on tumor cells and cell-mediated immunity was also studied.

## *Material and Methods*

The investigated population consisted of 135 normal subjects – (68 men and 67 women) divided into six approximately equal age groups from 20 to more than 70 years (mean age. 49.8 years) – and in 57 patients. 45 of them suffered from malignant evolutive lymphocytic proliferations with bone marrow infiltration (35 CLL and 10 well-differentiated LLS mean age: 63.9 years) and 12 were in remission after chemotherapy (1 CLL and 11 LLS, mean age: 63.2 years). The cases of LLS were defined according to the following criteria. (1) Proliferation of lymphocytes that appear to be mature with moderate variations in the nuclear size and shape (malignant lymphoma, well-differentiated lymphocytic, in Rappaport's [21] classification). (2) Enlargement of a single node or lymph node group in the early stage of the disease. (3) Extension of the malignant lymphoma to other lymph nodes and to different sites including mainly bone marrow spleen, liver and the gastrointestinal tract. (4) Hematologic findings such as anemia, normal or moderate increase of leukocytes with a high percentage of lymphocytes in the blood.

The lymphocytes were isolated from 10 ml of venous heparinized blood (either Liquemine, Roche, or Calparine, Choay 10 U/ml) by centrifugation (500 g for 20 min) at room temperature on a Ficoll-sodium metrizoate (Lymphoprep, Njegaard & Co Oslo, Norway) gradient. The isolated cells were then washed three times in Hanks balanced salt solution (HBSS) at pH 7.4 and spun at 200 g for 10 min. The nucleated cells were counted after lysis of the suspension in a mixture of Cetrimide (ICI) 20% (4 parts) and acetic acid glacial (1 part) and the cell viability was determined after conventional trypan blue staining (5.5% ± 0.3 of dying cells) The lym-

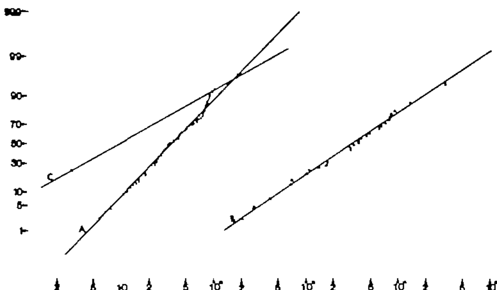



*Fig. 2.* Distribution of MRFC per mm³ in 135 healthy subjects (curve A), in 40 cases of evolutive CLL and generalized well-differentiated LLS (curve B) and in 12 cases in remission (curve C). Abcissa: logarithmic scale, number of rosettes per mm³ ordinate: probit scale, cumulative percentages. Small dots = Controls, ● = CLL, ▲ = LLS.

## *Discussion*

Our method of isolating lymphocytes and preparing the sheep rosettes from the normal blood gives results lower than those observed by several other authors [2, 12, 14 26] who have studied the total or late E rosettes. There are also similar differences between our data and those of STATHOPOULOS and ELLIOTT [25] who investigated SRFC and MRFC in CLL patients. On the other hand, our results (BF) looked similar to those reported by WYBRAN and FUDENBERG (WF) [29] using the 'active' or early E rosette technique (28.4 / ± 6.5). To show that our experimental conditions probably selected the subpopulation of T lymphocytes which are directly implicated in the cell-mediated immune process and are able to form 'active' rosettes with SRBC, we performed this rosetting test simultaneously by the two methods on blood lymphocytes of 34 normal and pathological subjects. The relationship between the percentages of SRFC

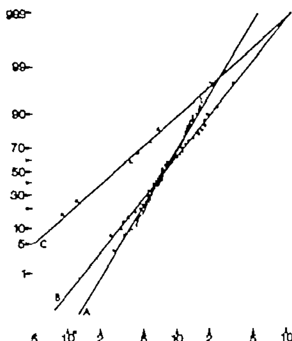



*Fig 1* Distribution of SRFC per mm³ in 135 healthy subjects (curve A), in 40 cases of evolutive CLL and generalized well-differentiated LLS (curve B) and in 12 cases in remission (curve C) Abcissa. logarithmic scale, number of rosettes per mm³ ordinate: probit scale, cumulative percentages. Small dots = Controls ●O = CLL (groups B and C, respectively) ▲ = LLS

numbers, showed a MRFC percentage in the range of control values with a normal absolute number and a normal SRFC/SRFC + MRFC ratio in two of them. On the other hand, the fifth case (G F ) presented a significant increase of the SRFC level

In the nonevolutive patients (group C) tested at least 3 weeks after cessation of chemotherapy the proportion of the two types of rosettes re turned to the normal range, whereas the lymphopenia caused a 50% de crease of their absolute number (table I II fig. 1 2) The SRFC/SRFC + MRFC ratio rose to the control values. The follow up of the patients demonstrated a progressive recovery of the RFC absolute number to normal levels during the subsequent months.

Figure 3 illustrates the variations of the lymphocytes, SRFC and MRFC in 2 patients suffering from CLL, the first 1 in relapse and the second 1 entering remission under chemotherapy In each case, the MRFC changes followed the modifications of the lymphoid cell numbers.

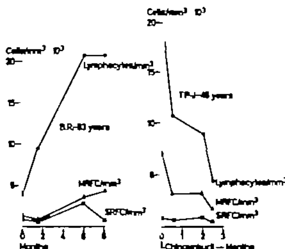



*Fig. 3.* Variations of lymphocytes, SRFC and MRFC per mm³ in 2 patients with CLL. On the left side: patient in relapse; on the right side: patient entering remission under chemotherapy.

*Table III.* Rosetting pattern of 5 peculiar cases of malignant lymphocytic proliferation

| Patient | Diagnosis | Sex and age | | Lymphocytes/mm | SRFC, % | SRFC/mm | MRFC, % | MRFC/mm³ | SRFC/SRFC + MRFC |
|---|---|---|---|---|---|---|---|---|---|
| G F | CLL | ♀ | 56 | 36,472 | 38.2 | 13,932 | 0.51 | 186 | 0.984 |
| L.L | CLL | ♀ | 56 | 20,458 | 6.28 | 1,285 | 0.25 | 51 | 0.962 |
| P A. | LLS | ♂ | 57 | 17,854 | 0.51 | 91 | 0.63 | 112 | 0.448 |
| T E. | LLS | ♂ | 44 | 5,215 | 3.46 | 180 | 0.44 | 23 | 0.887 |
| V A. | LLS | ♂ | 74 | 25,641 | 1.29 | 331 | 0.58 | 149 | 0.690 |

sonable to conclude that the two subpopulations isolated by both methods are identical. STATHOPOULOS and ELLIOTT [25] also found MRFC values higher than ours, but in more recent papers, GUPTA *et al.* [11] reported that the maximum of MRFC was obtained at temperatures between 24 and 28 C. The possible reason of our lower values could be due to the choice of the incubation temperature (37 C).

It is well known that the CLL is characterized by an important proliferation of B cells, which can be identified by surface immunoglobulins [1

*Table I.* SRFC and MRFC values in normal subjects and in patients suffering from either CLL or w differentiated LLS

| | Group A (135 controls) | | Group B (40 evolutive cases) | | Group C (12 non-evolutive cases) | |
|---|---|---|---|---|---|---|
| | geometric mean ± SD | median value (confidence interval 95 %) | geometric mean ± SD | median value (confidence interval 95 %) | geometric mean ± SD | median value (confide interval 95%) |
| SRFC, % | 29 (20.3–41.5) | 28.8 (14.2–58.6) | 4.8 (1.6–14.8) | 5.0 (0.7–34) | 20.7 (10.6–40.2) | 19.3 (3.8–96.5 |
| MRFC, % | 1 4 (0 6–3 1) | 1 4 (0.3–6.8) | 20.9 (10.6–41.5) | 20.3 (4 8–86.1) | 1.0 (0 4–3 1) | 0.7 (0.1 5 8) |
| SRFC/mm³ | 756 (445–1,285) | 750 (258–2,179) | 789 (352–1 766) | 771 (186–3 197) | 306 (126–741) | 275 (37 2,05 |
| MRFC/mm³ | 36 (15–85) | 35 (6–202) | 3,259 (850–1..500) | 3,021 (205–44,590) | 15 (4–58) | 9 (0.4–.36) |
| SRFC/SRFC + MRFC | 0.94 (0.90–0.99) | 0.94 (0 86–1.03) | 0.16 (0.05–0.54) | 0.15 (0.01–2.2) | 0.92 (0 79–1.06) | 0.95 (0.87 1 0 |
| Lymphocytes/mm³ | 2,610 (1 719–3,961) | 2,592 (1 115–6,027) | 16,490 (6,222–43 710) | 15,630 (2,151–113,600) | 1 479 (640–3 418) | 1,246 (283–5 49 |

*Table II.* Statistical comparison of groups A, B, C. (Student's t test on geometrical values)

| | Groups A/B | | Groups A/C | | Groups B/C | |
|---|---|---|---|---|---|---|
| | t | p | t | p | t | p |
| SRFC, % | 4.52 | < 0.001 | 2.89 | < 0.01 | 2.46 | < 0.02 |
| MRFC, % | 13.25 | < 0.001 | 9.81 | < 0.001 | 2.63 | < 0.02 |
| SRFC/mm³ | 0.39 | N.S. | 5.32 | < 0.001 | 3.50 | < 0 01 |
| MRFC/mm³ | 25.22 | < 0.001 | 3.11 | < 0.01 | 11 74 | < 0.001 |
| SRFC/SRFC + MRFC | 17.06 | < 0.001 | 1.39 | N.S. | 4.94 | < 0 001 |
| Lymphocytes/mm³ | 17.33 | < 0.001 | 4.08 | < 0.001 | 7 74 | < 0.001 |

obtained by the two techniques was estimated by calculating the following regression equation.

$$\text{Log SRFC BF\%} = 1.03179 \log \text{SRFC WF\%} + 0.04563 \quad (r = 0.83,\ p < 0.001).$$

Since the regression coefficient had a slope of almost 45° it seems rea

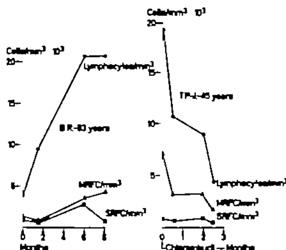



*Fig 3* Variations of lymphocytes, SRFC and MRFC per mm³ in 2 patients with CLL. On the left side: patient in relapse; on the right side: patient entering remission under chemotherapy

*Table III.* Rosetting pattern of 5 peculiar cases of malignant lymphocytic proliferation

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It is well known that the CLL is characterized by an important prolif eration of B cells, which can be identified by surface immunoglobulins [1

6 16 19 24 27] We tried to find out whether immunoglobulins were bound to the cytoplasmic membrane of the MRFC. The results obtained in 3 of our leukemic patients show that a high number of lymphoid cells isolated by the MRFC method, then subjected to a short lysis in distillated water still fixed a polyvalent heterologous fluorescein labelled antiserum directed against human immunoglobulins [22] These observations confirm the recent results of GUPTA *et al* [11] who identified the presence of membrane-bound IgM on lymphocytes forming rosettes with MRBC.

In almost all the cases studied, when the lymphoproliferative disease was in evolution, the relative and absolute numbers of MRFC rose, whereas the percentage of SRFC and the SRFC/SRFC + MRFC ratio dropped although the absolute number of SRFC remained normal These modifications were reversible after an efficient chemotherapy In certain active cases, an increase of the absolute number of SRFC was found with a percentage lower than the control values. These observations confirm those of RABIN and WHITESIDE [20] LANG *et al* [15] and AUGENER *et al.* [3] emphasizing the importance of expressing the results in absolute values to appreciate correctly the immune status of the patient.

This is exemplified by the behaviour of SRFC in untreated and treated CLL and LLS Our results show that in most cases, there is no real cellular immune deficiency before the institution of a cytotoxic treatment. Indeed, the absolute number of SRFC is identical in controls and untreated patients (respectively 756 and 789) This number drops after chemotherapy (306) This alteration of the immune capacity persists for a more or less long time after completion of the treatment.

Considering only the percentage of SRFC could have led to a misinterpretation of the cellular immune status In fact this percentage depends upon the number of blood lymphocytes which varies as a consequence of the treatment. Simultaneous decrease of the absolute number of SRFC and of blood lymphocytes can give an almost unchanged percentage of SRFC (29% in controls and 20 7% in treated patients)

In a healthy subject, the whole population of blood B cells is able to aggregate the erythrocytes of the *Macaca speciosa* monkey [18] but only 1 4–8 6% of the blood lymphocytes form rosettes with MRBC [7 8 10 11 25] This proportion is greatly enhanced in most cases of CLL and well-differentiated LLS with bone marrow infiltration possibly as a consequence of the disappearance of a repressive mechanism inhibiting the expression of a clone which has a limited growth in the normal subject.

The alterations of the rosetting tests which we have described were

observed in the great majority of the pathological cases studied. Nevertheless, in some cases the absolute MRFC number and the SRFC/SRFC + MRFC ratio were normal or moderately decreased. Four hypotheses can conceivably explain the dissociation between the leukemic status of the patients and the negativity of the rosette test with MRBC. The leukemic lymphocytes could be either: (1) B lymphocytes with bound surface immunoglobulins related to other classes than IgM and which are unable to form rosettes with MRBC, (2) B lymphocytes with bound surface immunoglobulins of the IgM class which have no receptors for MRBC, or have lost them, or masked them (3) T cells which have no receptors for SRBC, or have lost them, or masked them in one of our patients (G F), the T origin of the CLL can be suspected because of the high number of SRFC – this type of leukemia was observed four times in a series of 60 patients followed by NOWELL *et al* [17] or (4) null' lymphocytes which have no receptors for heterologous erythrocytes and no surface immunoglobulins.

In conclusion, it appears that in the patients suffering from lymphoproliferative disorders, such as CLL and well-differentiated LLS the double test of rosettes with sheep and mouse erythrocytes may be a simple test of some diagnostic value. The test seems to be most useful in (1) quantifying the proliferative capacity of the tumor cells (2) evaluating the possible cellular immune deficiency: (3) appreciating the effectiveness of chemotherapy and (4) following the patients during the remission period and perhaps predicting the relapse.

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# Heterozygous $\beta$-Thalassaemia with Normal Haemoglobin Pattern

## Haematologic, Haemoglobin and Biosynthesis Study of 4 Families

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*Key Words* Thalassaemia Haemoglobin pattern Genetics Biosynthetic studies

*Abstract* Thalassaemia with normal levels of Hb $A_2$ and Hb F and with an $\alpha/\beta$ ratio higher than 1 is described in 4 families. 3 of these families show direct or indirect signs of the presence of the $\delta$-thalassaemia gene along with the $\beta$-thalassaemia gene. The fourth family leaves the question as to whether there is a single mutation of the $\delta\beta$ tract or a $\beta + \delta$-thalassaemia in coupling unanswered. The necessity of knowing of the existence of this thalassaemia which conceals the presence of a $\beta$-thalassaemia gene, is stressed, above all in view of the danger that mating between a carrier of this thalassaemia and a carrier of classical $\beta$-thalassaemia could result in the birth of children with Cooley's disease.

The $\delta\beta$-thalassaemia described by ZUELZER *et al* [17] is characterized in the heterozygous state by a haematological picture typical of thalassaemia and a haemoglobin picture with a normal level of Hb $A_2$ and a level of Hb F of 5–10–20%, and in the homozygous state, a mild Cooley's disease with only HbF

Another kind of thalassaemia characterized by normal levels of Hb $A_2$ and Hb F occasionally reported in parents of subjects with Cooley's disease, has been considered by various authors as a $\delta\beta$-thalassaemia without the characteristic increase in the Hb F level [2, 9 13 15 16]

It is known, however that a form of thalassaemia with a normal haemoglobin pattern can also be the phenotypical manifestation of other thalassaemic genotypes for example, $\alpha$-thalassaemia, the association of

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$\beta$-thalassaemia with $\delta$-thalassaemia in *cis* or *trans* and $\beta$-thalassaemia complicated by severe iron deficiency

The determination of serum iron level and treatment with iron easily discriminate the latter condition. The study of haemoglobin synthesis *in vitro* permits $\alpha$-thalassaemia to be recognized, except in completely latent cases. Vice versa, the identification of $\beta + \delta$-thalassaemia is still difficult today Family studies make it possible, in fact, to recognize this condition if the two mutations are in repulsion and cases of separate transmission of the two genes are observed in the descendents, but this is not possible if the two mutations are in coupling [3] In this latter case, in fact, the two conditions segregate together because they are very closely linked.

Further genetical events, which have been suggested as a possible cause of thalassaemia with normal Hbs $A_2$ and F levels, are a displaced crossing-over between $\delta$- and $\beta$-genes whose recombinant product – though hybrid in structure – exhibits the same electrophoretic behaviour of the normal $\beta$-chain, and a deletion of both $\delta$- and $\beta$-genes [12]

In the present study 4 families carrying thalassaemia with these characteristics are described, 3 of which show evidence of carrying $\delta$- and $\beta$-thalassaemia genes and the other of which is more difficult to account for

## *Materials and Methods*

3 families came to our observation as the result of the presence of member with Cooley's disease. In 1, the proband was simply $\beta$-thalassaemia carrier On the whole the investigations concerned (table I fig. 1) 29 contactable relatives, amongst hom were the most significant members of each family

Routine haematological methods were used for the determination of haemoglobin, haematocrit and red cell count Red cell fragility was tested on 0.36% saline according to the method of SILVESTRONI and BIANCO [8]

Haemoglobin analysis was performed by micro-electrophoresis on cellogel in glycine-NaOH buffer at pH 8 6. Hb $A_2$ and Hb F were estimated by densitometric dosage carried out on the cellogel strip after electrophoresis according to the technique described by BIANCO *et al.* [1]. Hb F was measured by the method of SINGER *et al.* [11].

Globin chain synthesis was measured by incubation of peripheral blood in the presence of $^3$H-leucine (purchased from the New England Corp., Boston, Mass.) according to the technique of WEATHERALL *et al.* [14]. The total radioactivity incorporated into each globin chain was calculated as the sum of the radioactivity of all the effluent fractions of the globin chain peak. $\alpha/\beta$ or $\alpha/\beta+\gamma$ or $\alpha/\gamma$ ratios were calculated. The normal $\alpha/\beta$ ratio found in our laboratory based on 21 determinations performed on different subjects, was $1.00 \pm 0.011$.

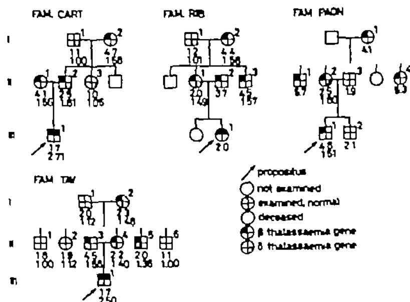



*Fig 1* Pedigrees of 4 families carrying thalassaemia with normal haemoglobin pattern. In 3 families members with Cooley's disease are present. The first number below the symbol of healthy subjects represents the percent of Hb $A_2$ the second number represents the $\alpha/\beta$ ratio. Below the symbol of the patients the percent of Hb $A_2$ and $\alpha/\beta+\gamma$ or $\alpha/\gamma$ ratio are reported.

## *Results*

*Cart family* The propositus (III 1) is a 2-year-old baby Sardinian, pallid and splenomegalic from the age of 6 months. Since then, he is in the care of the Center of Microcythaemia of Rome, for anaemia, and receives periodic transfusions. Haemoglobin electrophoresis shows a high percent of Hb A, but the study of globin synthesis shows the complete absence of newly forming $\beta$-chains (fig. 2). The parents (II 1 and II 2) are both carriers of evident thalassaemia, and show a high $\alpha/\beta$ ratio similar to that of $\beta$-thalassaemics (1.56 and 1.81) Haemoglobin electrophoresis, however reveals (table I) that the father (II 2) has a normal level of Hb $A_2$ (2.5) and Hb F (1 1) An examination of the family of this subject, resident in Sassari (Sardinia) whose blood samples were flown to Rome showed that the mother (I 2) is a carrier of $\beta$-thalassaemia with high Hb A (4 7%) and a high $\alpha/\beta$ ratio (1.53). Vice versa, the father (I 1) and a sister (II-3) have a normal haematologic picture and globine chain synthesis, but both have very low Hb A levels (1 1 and 1.0).

*Rib family* The propositus (III 1) is a 14-year-old girl from Venetia, in the care of the Center of Microcythaemia of Rome from the age of 3 months for anaemia with splenomegaly Since then, she receives transfusions at intervals of 20–30 days, despite the fact that at the age of 8 years splenectomy was performed. The parents are both carriers of evident thalassaemia, but while the father (II 2) has a high Hb

*Table I.* Haematologic, haemoglobin and chain synthesis data in 4 families possessing β- and δ-thalassaemia genes

| Family members | Age, years | RBC, $10^6$/mm | Hb, g/100 ml | Morphologic RBC aberrations | MCV C μ | Haemolysis, % in 0.36 % saline | Hb $A_2$ % | Hb F % | Globin chain synthesis ratios α/β | α/β+γ | α/γ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Fam. Cart* | | | | | | | | | | | |
| I-1 | 62 | 5.0 | 14.4 | – | 100 | 99 | 1.1 | 1.0 | 1.00 | | |
| I-2 | 57 | 5.2 | 11.6 | + + | 71 | 69 | 4.7 | 1.5 | 1.58 | | |
| II-1 | 33 | 5.2 | 12.6 | + + | 75 | 65 | 4.1 | 1.5 | 1.56 | | |
| II-2 | 31 | 5.4 | 11.6 | + + | 77 | 50 | 2.5 | 1.1 | 1.81 | | |
| II-3 | 28 | 4.8 | 11.2 | – | 87 | 99 | 1.0 | 1.1 | 1.05 | | |
| III-1 | 2 | 3.5 | 9.7 | + + + | 74 | 95 | 1.7 | 18.5 | | | 2.71 |
| *Fam. Rab* | | | | | | | | | | | |
| I-1 | 61 | 4.2 | 14.4 | – | 102 | 97 | 1.2 | 0.6 | 1.01 | | |
| I-2 | 66 | 4.5 | 11.6 | + + | 71 | 84 | 4.4 | 1.1 | 1.58 | | |
| II-1 | 35 | 5.2 | 12.6 | + | 69 | 75 | 2.0 | 1.4 | 1.49 | | |
| II-2 | 41 | 5.3 | 11.9 | + + | 79 | 69 | 3.7 | 2.1 | | | |
| II-3 | 27 | 6.0 | 12.8 | + + | 68 | 60 | 4.5 | 1.2 | 1.57 | | |
| III-1 | 14 | 3.2 | 9.3 | + + | 81 | 74 | 2.0 | 15.2 | | | |
| *Fam. Paon.* | | | | | | | | | | | |
| I-1 | 78 | 5.9 | 12.2 | + + | 65 | 80 | 4.1 | 2.3 | | | |
| II-1 | 50 | 6.0 | 12.8 | + + | 71 | 50 | 5.7 | 1.9 | | | |
| II-2 | 47 | 5.4 | 13.0 | + + | 70 | 55 | 2.5 | 1.5 | 1.80 | | |
| II-3 | 49 | 4.8 | 14.3 | – | 97 | 99 | 1.9 | 2.2 | | | |
| II-3 | 40 | 5.1 | 10.0 | + + | 75 | 60 | 5.3 | 2.8 | | | |
| III-1 | 19 | 5.4 | 11.6 | + + | 72 | 61 | 4.8 | 1.8 | 1.51 | | |
| III-2 | 17 | 4.7 | 12.8 | – | 97 | 99 | 2.1 | 2.3 | | | |
| *Fam. Tav.* | | | | | | | | | | | |
| I-1 | 70 | 4.6 | 12.8 | – | 100 | 99 | 2.0 | 1.2 | 1.12 | | |
| I-2 | 64 | 4.7 | 12.0 | | 85 | 95 | 2.3 | 1.1 | 1.45 | | |
| II-1 | 35 | 4.9 | 14.7 | – | 100 | 99 | 1.8 | 1.1 | 1.00 | | |
| II-2 | 32 | 5.0 | 12.8 | – | 96 | 99 | 1.9 | 1.1 | 1.12 | | |
| II-3 | 32 | 5.7 | 13.2 | + + | 77 | 82 | 4.5 | 1.4 | 1.58 | | |
| II-4 | 29 | 4.5 | 10.6 | + | 93 | 84 | 2.2 | 1.5 | 1.40 | | |
| II-5 | 27 | 5.0 | 14.0 | ± | 90 | 99 | 2.0 | 1.1 | 1.36 | | |
| II-6 | 23 | 4.7 | 13.6 | – | 94 | 98 | 1.9 | 1.1 | 1.00 | | |
| III-1 | 1 | 5.0 | 11.8 | + + | 76 | 84 | 1.7 | 15.8[a] | | 2.50 | |

The patients receive frequent transfusions.

The patient was given two transfusions, one 3 months and the other 1 month before the above examinations.

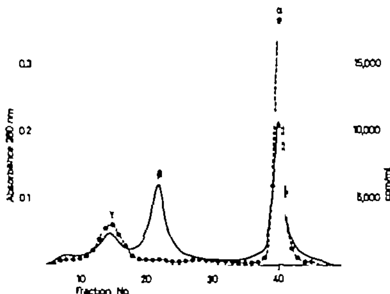



*Fig 2.* Distribution of radioactivity in the α- β- and γ-chains of a whole red blood cell lysate prepared from the patient with thalassaemia major of the Cart. family β-Chain synthesis is not detectable. The non-radioactive peak of β-globin is totally of transfusional origin. The α-chains are synthetized in a large excess over the γ-chains. — = Absorbance ··· = radioactivity

$A_2$ level (3.7), the mother (II 1) has always shown a normal Hb $A_2$ level (2 on average) on repeated examinations. Recently a study of this woman's family revealed that the mother (I 2) and a brother (II 3) are carriers of β-thalassaemia with a high Hb A level and a high α/β ratio, while the father (I 1) has a normal haematological picture and α/β ratio, but a very low Hb $A_2$ level (1.2).

*Paon family* This family has already been dealt with in a previous publication by SILVESTRONI *et al.* [10]. The study is now completed by the study of globin sy thesis *in vitro*

The proband is a 19 year-old youth, a β-thalassaemia carrier He has a high Hb A level (4.8) and an α/β ratio of 1.51 The father (II 3) and a brother (III 2) are normal. The mother (II 2) is a thalassaemia carrier with an α/β ratio of 1.80, but constantly presents normal Hb $A_2$ values (the mean of repeated examinations is 2.5) and Hb F levels (1.5). The study of this women's family reveals that the mother (I 1) and 2 brothers (II 1 and II-4) are carriers of β-thalassaemia with a high Hb $A_2$.

*Tar family* The propositus is a 1-year-old child from Puglia, pallid from the age of 2 months. He had already received two transfusions, one 3 months and the other 1 month before the present examinations. An electrophoretic study of the Hb reveals the presence of a high quantity of Hb A which does not, however all come from the transfused blood. The study of globin synthesis *in vitro* in fact, reveals that a notable quantity of β-chains are synthesized by the patient's own reticulocytes (fig. 3) The father (II 3) of the child is an evident β-thalassaemia carrier Vice ver

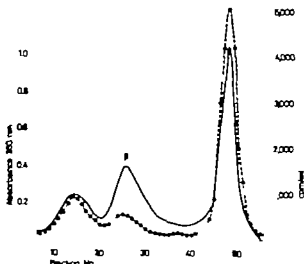



*Fig 3* Distribution of radioactivity in the α-, β- and γ-chains of a whole red blood cell lysate prepared from the patient with thalassaemia major of the Tav family A notable β-chain synthesis can be seen. A large excess of chain over combined β+γ-chain radioactivity is present. — = Absorbance = radioactivity

in, in the mother (II 3) the thalassaemic haematological characteristics are almost entirely absent, the haemoglobin picture is normal and only the study of globin synthesis reveals deficit in α-chains (α/β ratio = 1.40), similar to that of β-thalassaemia carriers. The mother of this subject (I 2) and brother (II 5) present identical haematological, haemoglobin and globin synthesis characteristics, while the father (I-1) and another 3 brothers (II 1 2, 6) are normal.

## *Discussion*

In the 3 families having a member with Cooley's disease (Cart., Rib. and Tav ) 1 of the 2 parents is a thalassaemia carrier with a high Hb $A_2$ and the other a thalassaemia carrier with normal values of Hb A and Hb F but with α/β ratios quite similar to those found in heterozygous β-thalassaemics. This finding together with the fact of there being a child with Cooley's disease reveals the presence in these 3 subjects of a β-thalassaemia gene, or better given the absence of an increase in Hb $A_2$, of a thalassaemic mutation at locus β and at locus δ.

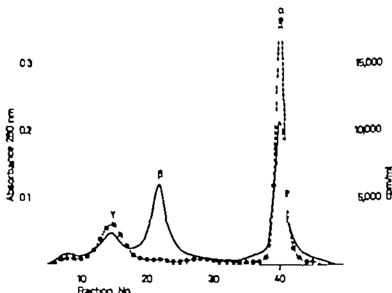



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can result in offspring with Cooley's disease. The present case material clearly illustrates this danger: 3 of the families dealt with have come to our observation after the birth of a child with Cooley's disease.

This all points to the necessity of thoroughly studying every case of thalassaemia with a normal haemoglobin picture, above all if marriage with a β-thalassaemia carrier is in view, and also indicates the necessity of including in these investigations the study of globin synthesis *in vitro* which is, at present, the only investigation which makes it possible to detect a thalassaemic mutation at locus β or on the δβ tract, even in the absence of any other haematological or haemoglobin thalassaemia characteristic.

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Even in the third family with a Cooley's patient (Tav family) a $\beta$-thalassaemia and a compromission of the $\delta$-chains synthesis ($\delta$-thalassaemia) are present but in this case these two genetic conditions are in coupling, since they are transmitted together by the double carrier (either none of them as to II 1 2 6 or both of them as to II-4 5 and, according to the here proposed genetic interpretation III 1) The basis for such failure of both $\beta$ and $\delta$-chains synthesis by the same non $\alpha$-genes cluster remains, however not identified the present findings being in fact, compatible both with a single genetic event responsible for both the abnormalities (a Lepore gene electrophoretically indistinguishable from a $\beta$-gene or a $\delta$- and $\beta$-genes delection see Introduction and MODIANO *et al* [6]) and with two independent mutations in *cis* one responsible for the $\beta$ and the other for the $\delta$-thalassaemia

A more remote possibility that should be considered if a single genetic event is at the basis of the simultaneous $\beta$- and $\delta$-chains synthesis compromission with no increase of $\gamma$-chains synthesis (as in the typical $\delta\beta$-thalassaemias) is that of a displaced crossing-over between the left test of the $\gamma$ genes (a $^{G}\gamma$-gene see RICCO *et al* [7]) and the $\beta$-gene such that the resulting hybrid gene would produce globin chains electrophoretically identical to $\beta$-chains but in a reduced quantity (= $\beta$-thalassaemia) This hypothetical cluster would consist only of that hybrid gene and it would therefore produce only those globin chains electrophoretically indistinguishable from the $\beta$-chains The phenotype associated with it should then be $\gamma$-thalassaemia during the fetal life and no HPFH during the extrauterine life (while, when the $^{A}\gamma$-gene is the one involved in the displaced crossing-over with the $\beta$ gene an HPFH phenotype of the $^{G}\gamma$-type ensues [5]) $\gamma$-thalassaemia and insufficient production of $\beta$-chains (= $\beta$-thalassaemia) during the post-natal life A form of $\gamma$-thalassaemia (high $\alpha/\gamma$ synthetic ratio) which after the birth, gradually became a $\gamma + \beta$-thalassaemia was indeed described [4] It might have had this origin The $\delta$- and $\beta$-thalassaemias in coupling of family Tav may also be due to this type of displaced crossing-over but, in order to consider this explanation more likely the finding of an associated $\gamma$-thalassaemia would be required.

Along with the theoretical aspect of the knowledge of this particular form of thalassaemia, another important practical point is to be reported that is, the necessity of knowing that the $\beta$ thalassaemia gene can also be present in a thalassaemia carrier with a normal Hb $A_2$ value and that mating between a carrier of this thalassaemia and a $\beta$-thalassaemia carrier

can result in offspring with Cooley's disease. The present case material clearly illustrates this danger 3 of the families dealt with have come to our observation after the birth of a child with Cooley's disease.

This all points to the necessity of thoroughly studying every case of thalassaemia with a normal haemoglobin picture, above all if marriage with a $\beta$-thalassaemia carrier is in view and also indicates the necessity of including in these investigations the study of globin synthesis *in vitro* which is, at present, the only investigation which makes it possible to detect a thalassaemic mutation at locus $\beta$ or on the $\delta\beta$ tract, even in the absence of any other haematological or haemoglobin thalassaemia characteristic.

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Acta haemat. 59: 341–347 (1978)

# Interaction of Hb $A_2$ Indonesia Trait with β-Thalassaemia Trait and with Hb E Trait[1]

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*Key Words.* Hb $A_2$ Indonesia · β-Thalassaemia · Hb E

*Abstract.* The haematological findings resulting from the interaction of Hb $A_2$ Indonesia trait with β-thalassaemia trait and $HbA_2$ Ind trait with Hb E trait are discussed. A person doubly heterozygous for Hb $A_2$ Ind and β-thalassaemia had mild haematological abnormalities essentially similar to those found in persons with β-thalassaemia trait alone. A carrier for both Hb $A_2$ Ind trait and Hb E trait had essentially normal haematological findings.

Abnormal haemoglobins and thalassaemia have been shown to be an important cause of hereditary haemolytic anaemias in Malaysia and in several other countries in Southeast Asia. Hb $A_2$ Indonesia (Hb $A_2$ Ind) is a δ-chain variant in which the glycine residue in position 69 is replaced by an arginine residue [5]. This haemoglobin has been observed in appreciable frequencies in Indonesians from Sumatra [3, 4] and it has also been found in Malaysia [1, 2] among Malays, particularly among descendants of migrants from Sumatra, and also among the West Malaysian aborigines. In this paper we report the haematological findings resulting from the interaction of Hb $A_2$ Ind trait with β-thalassaemia trait and Hb $A_2$ Ind trait with Hb E trait.

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## Methods

Haematological examinations were carried out according to standard methods. Haemolysates were prepared from washed packed red cells by the addition of 1 vol of water and 0.5 vol of toluene. Haemoglobin F levels were measured by the method of SINGER *et al* [7]. Electrophoresis of haemoglobin was done on starch gel using Tris-EDTA boric acid buffer at pH 8.6 and discontinuous Tris-boric acid buffer at pH 9.5. Cellulose acetate electrophoresis was done in Tris-EDTA-boric acid buffer at pH 8.9. Haemoglobin A and other haemoglobin components were quantitated by the cellulose acetate electrophoretic method of MARENGO-ROWE [6].

## Case Reports

*Family A*

A 1 year-old female Malay child was admitted to the General Hospital of Seremban with a history of pallor, cough and fever that had lasted for 1 week. On examination this child was found to be anaemic with a slight tinge of jaundice. The spleen was just palpable. Haematological investigations and haemoglobin analysis showed that this child had $\beta$-thalassaemia major. Subsequently, the parents of this child and other siblings were examined. The father was a Malay from Johore and the mother was also a Malay but she was not sure where her parents originally came from. Unlike the child the parents had no positive clinical findings. The haematological findings for the child (the youngest in the family) and the parents are shown in table I. The peripheral blood film of the child showed moderate hypochromia and anisopoikilocytosis of the red cells with some target cells and normoblasts (9/100 white cells). In the peripheral blood film of the father the red cells were fairly well haemoglobinised but showed mild anisopoikilocytosis with some target cells and microcytes. In the peripheral blood film of the mother the red cells showed mild hypochromia and anisopoikilocytosis with many elliptocytes and some microcytes and target cells. The total white cell count, the platelet count and the Motulsky's test for G6PD deficiency were normal in all 3 persons. From the findings shown in table I, it can be seen that the father had $\beta$-thalassaemia trait while the mother had Hb $A_{2\,Indonesia}$ in addition to $\beta$ thalassaemia trait. Seven other children in this family were also examined and four of them had Hb $A_{2\,Indonesia}$ trait only, while the other 3 had only $\beta$-thalassaemia trait. The haematological findings for these children are not given in table I because several of them had superimposed mild iron deficiency. The starch gel electrophoretic patterns of the whole family at pH 8.6 are shown in figure 1.

*Family B*

This Malay family lived in the Ulu Jempul district of Kuala Pilah. Like most of the Malays in the Ulu Jempul district, the family descended from Minangkabau migrants from Sumatra. The youngest (fourth) child in this family was investigated for anaemia at the District Hospital, Kuala Pilah. Subsequently, all members of the family were examined. Several of the family members had iron deficiency (confirmed by serum iron estimation) and some also had megaloblastic anaemia. Clinically, except

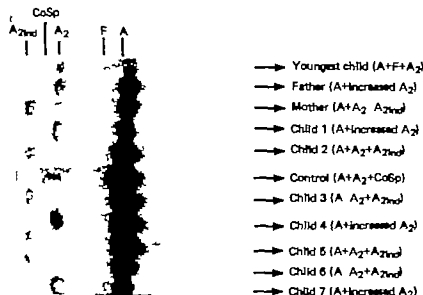



*Fig 1* Starch-gel electrophoresis in Tris-EDTA-boric acid buffer pH 8 6 showing the haemoglobin patterns of the members of family A

for the pallor there were no positive findings The members of the family were given oral iron and folic acid, and subsequently 11 of them were re-examined. Despite treatment the mother was still iron-deficient the last time she came for follow-up The last available haematological findings for the mother shown in table II, showed mild abnormalities which are most probably the result of iron deficiency The haematological findings for the father and the first 3 children are also shown in table II. The peripheral blood film findings of the father and these 3 children were essentially within normal limits Starch gel electrophoretic patterns of this family at pH 8 6 are shown in figure 2 It can be seen from table II and figure 2 that child 1 in this family had both Hb E trait and Hb $A_{2\,Ind}$ trait. The fourth child in this family is not listed in table II This child also had Hb E trait and Hb $A_{2\,Ind}$ trait (fig 2) but this child, like the mother still showed some iron deficiency the last time she came for follow-up hence, could not determine the haematological findings after her iron deficiency corrected

## *Discussion*

In family A the father had $\beta$-thalassaemia trait while the mother had Hb $A_{2\,Ind}$ trait in addition to $\beta$-thalassaemia trait. The findings in the

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*Table II* Haematological findings of family B

| | Age, years | Hb, g/100 ml | RBC ml/mm | PCV | MCV | MCH | MCHC | Reticulocytes, % | RBC morphology | Osmotic fragility | Hb pattern | Hb F % | Hb $A_2$ % | Hb $A_{2\,Ind}$ % | Hb E, % | Serum bilirubin, mg/100 ml | Serum Iron, µg/100 ml | TIBC µg/100 ml |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Father | 59 | 15.4 | 5.1 | 45.0 | 88.2 | 30.2 | 34.2 | 0.6 | 0 | normal | A + A + $A_{2\,Ind}$ | 0.9 | 2.0 | 1.2 | | 0.3 | | |
| Mother | 34 | 11.4 | 5.7 | 37.5 | 65.8 | 20.0 | 30.4 | 0.6 | + | normal | A + E | 1.4 | | | 27.9 | 0.7 | 32 | 426 |
| Child 1 (propositus) | 12 | 13.1 | 5.6 | 40.0 | 71.4 | 23.4 | 32.8 | 1.4 | 0 | normal | A + E + $A_{2\,Ind}$ | 1.1 | | 1.3 | 26.3 | 0.1 | | |
| Child 2 | 9 | 13.4 | 5.0 | 41.0 | 82.0 | 26.8 | 32.7 | 1.0 | 0 | normal | A + E | 1.1 | | | 26.0 | | | |
| Child 3 | 6 | 13.0 | 4.4 | 41.0 | 93.2 | 29.5 | 31.7 | 0.8 | 0 | normal | A + A + $A_{2\,Ind}$ | 1.0 | 1.9 | 1.4 | | 0.1 | | |

*Table I* Haematological findings of family A

| | Age, years | Hb, g/100 ml | RBC, ml/mm³ | PCV % | MCV | MCH | MCHC | Reticulocytes, % | RBC morphology | Osmotic fragility | Hb pattern | Hb F % | Hb $A_2$, % | Hb $A_{2\,Ind}$, % | Serum bilirubin mg/100 ml | Serum iron, µg/100 ml | TIBC, µg/100 ml |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mother (propositus) | 38 | 11.6 | 5 4 | 39.0 | 72.2 | 21.5 | 29 7 | 1.2 | | decreased | A+$A_2$ +$A_{2\,Ind}$ | 1 4 | 3.8 | 2.5 | 0.4 | 111 | 267 |
| Father | 5 | 12.8 | 4.9 | 40.5 | 82.7 | 26.1 | 31 6 | 0 4 | | decreased | A+$A_2$ | 0.7 | 4 4 | – | 0 4 | 103 | 315 |
| Youngest child | 1 | 8.4 | 3 9 | 29 0 | 74 4 | 21.5 | 29.0 | 2.2 | | decreased | A+F+A | 34.5 | 3 6 | – | – | – | – |

haematological findings in that person were not reported, although Lie-Injo stated that she had evidence that the gene for Hb $B_2$ ($A_2$ Ind) interacted with the gene for β-thalassaemia trait, leading to mild haematological abnormalities. The haematological findings in our subject doubly heterozygous for Hb $A_2$ Ind and β-thalassaemia also showed mild haematological abnormalities, but these are no more severe than those found in persons with *simple β-thalassaemia trait alone*.

While Hb $A_2$ Ind is due to a point mutation in the δ structural gene, which is closely linked to the β structural gene, the molecular basis for β-thalassaemia has not yet been elucidated and it is clear that there are different types of β-thalassaemia.

In family B child 1 had both Hb E trait and Hb $A_2$ Ind trait, but this double heterozygous state for Hb E and Hb $A_2$ Ind caused no haematological changes in the child.

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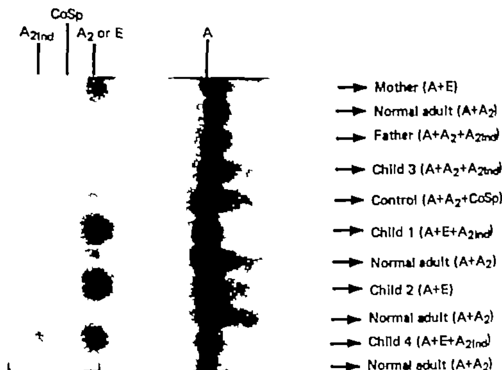



*Fig 2* Starch-gel electrophoresis in Tris-EDTA-boric acid buffer pH 8.6 showing the haemoglobin patterns of the members of family B

mother show that in a person who has Hb $A_{2\ Ind}$ trait in addition to β-thalassaemia trait, the haematological findings are essentially similar to those found in patients with β thalassaemia trait alone A point of diagnostic importance is that when Hb $A_{2\ Ind}$ trait is present in addition to β-thalassaemia trait, the diagnosis of β thalassaemia trait must not be missed. As discussed by LIE INJO *et al* [5] in persons heterozygous for Hb $A_{2\ Ind}$ the percentages of Hb $A_2$ and Hb $A_{2\ Ind}$ range from 1 3 to 2.1 and 1 0 to 1 4 respectively But in our propositus who had β thalassaemia trait in addition to Hb $A_{2\ Ind}$ heterozygosity the percentages of Hb $A_2$ and Hb $A_{2\ Ind}$ are roughly doubled and together equal the value of Hb A in β-thalassaemia trait This finding supports the relationship of allelism that must exist between Hb $A_2$ and Hb $A_{2\ Ind}$ as shown to exist between Hb A and other δ-chain variants, e g Hb $A_2$ or Hb $B_2$. LIE INJO reported finding a person who had Hb $A_{2\ Ind}$ in addition to β-thalassaemia trait in whom the values of Hb $A_2$ and Hb $A_{2\ Ind}$ were also roughly doubled compared to the levels found in persons with Hb $A_{2\ Ind}$ trait alone [5] The

leukocytes of patients with congenital methemoglobinemic syndrome without neurological symptoms [1, 10, 11, 16].

The present report describes another case of NADH-diaphorase deficiency associated with a progressive and severe neurological disease in a Spanish girl.

## *Materials and Methods*

Materials and methods have been described in detail before [10]. Briefly, the following methods were used: Van Kampen and Zijlstra [21] for methemoglobin determination, Hegesh *et al.* [4] for methemoglobin reductase activity in both erythrocytes and leukocytes, Kaplan and Beutler [12] for electrophoresis of NADH-diaphorase on starch-gel, and Beutler [2] for the other red cell enzyme determinations. Leukocytes were homogenized in 0.5% sodium deoxycholate and assayed for NADH-cytochrome $b_5$ reductase as described by Mihara and Sato [18]. The reduction of 10 $\mu M$ cytochrome $b_5$ was followed at 556 nm in the presence of 180 $\mu M$ NADH, and the activity expressed as the first order rate constant ($min^{-1}$) per milligram protein.

## *Case Report*

The propositus was a 2-year-old Spanish girl. She was born after an uneventful pregnancy. At birth, cyanosis of her lips, nail beds and oral mucosa was noted. At 3 months of age, she showed persistent and generalized cyanosis. At that time, she had hyperactive reflexes, generalized hypertonia and attitude in opisthotonos. Occasional athetoid movements of both extremities were also noted. Oxygen administration increased the neurological disturbances. Neither cardiac nor respiratory involvement could be found to account for the cyanosis. Methemoglobin was not measured at that time and the disorder was not recognized. At the age of 6 months, the girl showed intense and generalized cyanosis, severe mental retardation with microcephaly and growth retardation. Her EEG was normal. The methemoglobin concentration in her erythrocytes was found to be 28%. Hemoglobin electrophoresis at pH 8.6 was normal and alkaline-resistant hemoglobin (HbF) was present in normal amounts. No hemoglobin M was found on spectral analysis of the hemolysate and by hemoglobin electrophoresis at pH 7.2 on acetate cellulose strips.

At the age of 2 years, the girl's hematological data showed no substantial abnormalities (table I). Therefore, the diagnosis of congenital methemoglobinemia was established. The metabolic defect in the propositus was found to be an absence of NADH-diaphorase activity in her erythrocytes and leukocytes (table II). Traces of NADH-diaphorase with normal mobility were found by starch-gel electrophoresis of the hemolysates from the propositus. The activity of some erythrocyte glycolytic enzymes were found to be elevated in accordance with the age of the propositus.

She was treated with ascorbic acid (500 mg orally and daily) and methylene blue (1–2 mg/kg orally and daily). A marked reduction of methemoglobin content (up to 5% of normal) and cyanosis occurred, but her neurological symptoms persisted.

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# Congenital Methemoglobin Reductase (Cytochrome $b_5$ Reductase) Deficiency Associated with Mental Retardation in a Spanish Girl

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*Key Words.* Methemoglobinemia Mental retardation Cytochrome $b_5$ reductase Enzymopathy

*Abstract* Methemoglobinemia and mental retardation associated with NADH diaphorase deficiency was found in a 2 year-old girl of Spanish origin. She showed no NADH-diaphorase activity in either erythrocytes or leukocytes, but electrophoretic studies of the hemolysate showed traces of an enzyme with normal mobility Cytochrome $b_5$ reductase activity was also found to be absent in the leukocytes of the propositus. Intermediate NADH-diaphorase activity was found in erythrocytes and leukocytes in her parents and her sister in accordance with the autosomal recessive mode of inheritance of this enzymopathy The relationship between a generalized cytochrome b reductase deficiency and the progressive neurological involvement in our patient is discussed briefly

## *Introduction*

Deficiency of red blood cell enzyme NADH-diaphorase (methemoglobinreductase) leads to congenital methemoglobinemia [8 20] In some cases the methemoglobinemic syndrome is associated with a progressive neurological disorder retardation and death at a young age [1 3 5 8–10 15 16] Some of us (J C. K. and A L.) found NADH-diaphorase deficiency in the leukocytes of patients with congenital methemoglobinemia and neurological disease whereas nearly normal levels were found in the

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*Family Studies*

Both parents and the 3 year-old sister of the propositus had decreased erythrocy NADH-diaphorase activity (table II) without clinical or hematological abnormal ties; both parents showed normal methemoglobin content (less than 2% ) and nor mal electrophoretic pattern of NADH-diaphorase NADH-cytochrome $b_5$ reductase activity of the leukocytes studied only in the propositus and her mother is shown in table III.

## Discussion

Congenital NADH-diaphorase deficiency also called hereditary met hemoglobinemia, is a relatively rare disorder In most cases, it is a benign enzymopathy characterized by cyanosis, a variable degree of methemoglobinemia and compensatory polycytemia [8] However in about 15 % of the cases the syndrome is associated with a progressive neurological disorder and severe mental retardation [1 3 5 8–10 15 16] Ascorbic acid or methylene blue given orally are effective therapeutic agents for the reduction of methemoglobinemia and thus cyanosis, but they have no effect on the neurological disease In the present case for example, cyanosis and methemoglobinemia were markedly reduced by oral administration of ascorbic acid and methylene blue given together However neurological disease was progressive and at the time of our study the propositus could not maintain her head erect and a generalized hyperreflexia with hyper tonia was observed. Convulsive attacks of spasticity with athetoid move ments were also frequent. Recently some of us reported a case of met hemoglobinemia associated with severe mental retardation in a 6-year-old boy of Spanish origin [1] The neurological syndrome was very simila to that observed in the case reported here. The frequency of methemoglobin reductase deficiency in Spain has not been studied, but there appears to be a higher occurrence of this disease in populations originating from the med'terranean basin [KAPLAN *et al* unpubl ]

Although no obvious consanguinity between the parents, both heterozygotes, could be found, it is possible that the propositus is homozygous for a single abnormal allele

The relationship between methemoglobinemia and severe neurological impairement was poorly understood until the finding that the leukocytes from such patients are also deficient in diaphorase [1 10 11 16] That the defect is indeed generalized was recently proven in 1 case [16]

To date including the present case the leukocytes were found NA DH-diaphorase-deficient in 8 cases of methemoglobinemia with neurolog

*Table I* Hematological data of the propositus at the age of 2 years

| | |
|---|---|
| Red blood cells | $5.6 \times 10^{12}$/l |
| White blood cells | $17.5 \times 10$ /l |
| Platelets | $270.0 \times 10^{9}$/l |
| *Reticulocytes* | *95.[illegible] × 10 /l* |
| Hemoglobin | 11.3 g/dl |
| Packed red cell volume | 0.38 (l/l) |
| Mean red cell volume | 68.0 fl |
| Mean red cell hemoglobin | 23.0 pg |

*Table II* NADH-diaphorase activity of erythrocytes and leukocytes in the propositus, her family and in normal subjects

| | Erythrocytes | Leukocytes |
|---|---|---|
| Propositus | 0 | 0 |
| Mother | 1.73 | 61 |
| Father | 1.50 | – |
| Sister | 1.05 | 46 |
| Normal values | [illegible].8–3.9 | 70–110 |

*Erythrocyte NADH-diaphorese activity is expressed as micromoles of reduced sub*strate per gram hemoglobin at 37 C. Method of HEGESH *et al.* [4]

Leukocyte NADH-diaphorase activity is expressed as nanomoles of reduced substrate per milligram of protein at 37 C.

*Table III* NADH-cytochrome $b_5$ reductase activity of the leukocytes

| Subject | Activity (first-order rate constant per mg of protein) |
|---|---|
| Propositus | not detectable |
| Mother | 1.62 |
| Control | 2.04 |
| *Normal range* | *3.20 ± 0.80* |

The activity was measured in deoxycholate-treated homogenates as described in the text by using cytochrome b as a final electron acceptor. This method precludes any direct measurement in red cell lysates because of the interference of hemoglobin. On the contrary *it can be applied to crude leukocytes extracts.*

*Family Studies*

Both parents and the 3 year-old sister of the propositus had decreased erythrocyte NADH-diaphorase activity (table II) without clinical or hematological abnormalities; both parents showed normal methemoglobin content (less than 2%) and normal electrophoretic pattern of NADH-diaphorase. NADH-cytochrome $b_5$ reductase activity of the leukocytes studied only in the propositus and her mother is shown in table III.

## Discussion

Congenital NADH-diaphorase deficiency also called hereditary methemoglobinemia, is a relatively rare disorder. In most cases, it is a benign enzymopathy characterized by cyanosis, a variable degree of methemoglobinemia and compensatory polycytemia [8]. However in about 15 of the cases the syndrome is associated with a progressive neurological disorder and severe mental retardation [1 3 5 8–10 15 16]. Ascorbic acid or methylene blue given orally are effective therapeutic agents for the reduction of methemoglobinemia and thus cyanosis, but they have no effect on the neurological disease. In the present case for example cyanosis and methemoglobinemia were markedly reduced by oral administration of ascorbic acid and methylene blue given together. However neurological disease was progressive and at the time of our study the propositus could not maintain her head erect and a generalized hyperreflexia with hypertonia was observed. Convulsive attacks of spasticity with athetoid movements were also frequent. Recently some of us reported a case of methemoglobinemia associated with severe mental retardation in a 6-year-old boy of Spanish origin [1]. The neurological syndrome was very similar to that observed in the case reported here. The frequency of methemoglobin reductase deficiency in Spain has not been studied, but there appears to be a higher occurrence of this disease in populations originating from the mediterranean basin [KAPLAN *et al.* unpubl.].

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To date, including the present case the leukocytes were found NADH-diaphorase-deficient in 8 cases of methemoglobinemia with neurolog

ic involvement [13] All these patients displayed a similar clinical pattern with cyanosis associated with athetosis, microcephaly and severe mental retardation In contrast in 10 patients with the benign type of congenital methemoglobinemia only red cells were found defective with normal or subnormal levels of NADH-diaphorase in leukocytes [13]

Actually the so-called methemoglobin reductase or NADH-diaphorase is identical to NADH-cytochrome $b_5$ reductase (EC 1 6.2.2) [7 17] Subjects with red cell NADH-diaphorase deficiency are also defective in NADH-cytochrome $b_5$ reductase [10 14 16] Therefore it is now clear that there are two clinical types of disease type I in which the only symptom is a well tolerated methemoglobinemia, and type II in which the methemoglobinemic syndrome is associated to a progressive encephalopathy

The enzyme defect in type I is a deficiency of red cell soluble cytochrome $b_5$ reductase In type II there is a generalized defect of both soluble and microsomal cytochrome $b_5$ reductase [16] The present case belongs to the type II category and as expected, the leukocytes were found deficient in both NADH-diaphorase and cytochrome $b_5$ reductase [table II III] The impact of generalized cytochrome $b_5$ reductase deficiency upon the brain metabolism is still not elucidated. However since the cytochrome $b_5$ system plays a major role in the desaturation of fatty acids [6 19] the neurological symptoms observed in type II disease might be due to a defective metabolism of lipids in the nervous tissue

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# A 13-Years Remission in Chronic Myelocytic Leukemia after a Single Course of Busulfan

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*Key Words.* Busulfan · CML · Prolonged remission

*Abstract.* A patient with chronic myelocytic leukemia in whom a clinical and hematological remission occurred after a single course of busulfan is described. The remission lasted for a period of 13 years and during that lapse of time, she gave birth to a healthy child. Afterwards, acute myeloblastic leukemia was diagnosed. Following treatment with rubidomycin and arabinoside C another remission was achieved, lasting already for 18 months.

Prolonged remission in chronic myelocytic leukemia (CML) following a single course treatment with busulphan is rare [1]. We here describe a patient with CML in whom following a single course of busulphan, a remission of 13 years duration has been achieved.

## *Case Report*

In May 1962, a 27 year-old housewife, mother of a 2-month-old infant was referred to our hematological clinic because of weakness and leukocytosis. Her past history was irrelevant. The pregnancy was uneventful and she gave birth to a healthy child. Shortly afterwards she started to complain of vague joint pains and fatigue. On physical examination, the only phathological finding was a firm and nontender spleen, felt 1 cm below the costal margin. The hemoglobin was 12.2 g/dl, the white blood cell count 28,000/μl with 2% metamyelocytes, 4% myelocytes, 4% band forms, 65% polymorphonuclears, 2% eosinophils, 3% basophils, 4% monocytes

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and 16% lymphocytes. The platelet count was 300,000/µl. The bone marrow aspiration biopsy revealed marked hypercellularity with myeloid-erythroid ratio of 3.4/1. A marked hyperplasia of the bone marrow white blood cell series was noted, with 3.2% myeloblasts, 6% promyelocytes, 43.6% myelocytes, 27.4% metamyelocytes, 13.4% band forms, 6.6% polymorphonuclears and 0.8% monocytes.

CML was diagnosed and treatment with busulphan, 2 mg/day was started. 1 month later the patient condition improved and the spleen was not palpable anymore. The white blood cell count was 9,900/µl and no metamyelocytes and myelocytes were seen in the peripheral blood smear. The dosage of busulphan was gradually reduced and at the end of November 1962, about 7 months after the beginning of treatment and total dose of 200 mg busulphan, the drug was stopped. The patient's condition was satisfactory and there were no pathological findings on physical examination. The hemoglobin was 12.6 g/dl, the white blood cell count 6,000/µl with normal differential count and the platelet count was 250,000/µl. The bone marrow aspiration biopsy was normal. No cytogenetic study was performed.

In April 1965, following normal pregnancy the patient gave birth to healthy child. Until June 1975 the patient was examined every 3 months and remained in complete clinical and hematological remission. Repeated peripheral blood counts and aspiration bone marrow biopsies were within normal limits. The serum vitamin $B_{12}$ level was between 640 and 700 pg/ml and the neutrophile alkaline phosphatase (NAP) score was between 68 and 78 (normal 30–70). In June 1975, the patient was hospitalized because of severe low back pain lasting for 3 weeks. On admission she was acutely ill and unable to perform the slightest movement, because of unbearable back pain. The lymph glands were not enlarged and the spleen was not palpable. Marked tenderness was noted over the lumbar spine and pelvic regions. The hemoglobin was 13.4 g/dl, the white blood cell count 6,200/µl, with 17% myeloblasts, characterized by the presence of azurophilic granules and the platelet count was 220,000/µl. The NAP score was 304 serum B level 747 pg/ml. The electron microscopic examinations of the peripheral white blood cells confirmed the presence of typical myeloblasts. The bone marrow was hypercellular and revealed a marked proliferation of myeloblasts, no Philadelphia chromosome was found on karyotype examination. The urine and the routine biochemical examinations were normal except for increased urinary calcium excretion of 700 mg/24 h (normal up to 250 mg/24 h). The X-rays bone survey and the $^{99}Tc_m$ diphosphonate bone scan disclosed multiple osteolytic lesions in the lumbar spine and in the pelvis. Acute myeloblastic leukemia (blastic transformation) was diagnosed. Two 5-day courses of intravenously administered rubidomycin 60 mg daily and cytosine arabinoside 150 mg daily were given, with 5-day rest interval. A marked improvement in the intensity of her bone pains was noted. The urinary calcium excretion returned to normal values (170 mg/24 h). The treatment was followed by aplasia of the bone marrow complicated by fulminant enterococcal sepsis. Massive doses of antibiotics, whole blood, platelets and leukocytes transfusions brought to progressive improvement in the patient's condition. Simultaneously the hematological values gradually returned to normal and the patient entered into remission. She was discharged while on thioguanine 80 mg/day and to the present day i.e. 1 / years later the patient is still in remission and free from pains. The periferal blood counts and bone marrow exami-

nation are normal. The X-rays bone survey and the $^{99m}$Tc$_N$ bone scan are similar to those found 1½ years ago.

## Discussion

The diagnosis of CML, established in 1972 in this patient, was based upon the findings of splenomegaly leukocytosis and the characteristic morphological findings in the peripheral blood and in the bone marrow The achievement of a remission lasting for 13 years following a single and relatively short course of busulphan treatment is unusual The normal NAP scores and serum vitamin $B_{12}$ levels found in our patient at the numerous follow up examinations during this period of time are also in favor of the fact that she was in complete remission. It is to be noted that these parameters were not examined in 1962, when the diagnosis of CML was established Prolonged busulphan-induced remissions have been reported in CML [6 8 10 14 15] however similar results following a single course of busulphan treatment are rare. DJALDETTI *et al* [3] described a 42 year-old woman in whom complete remission of 7 years duration was achieved following a single course of busulphan. Another case of 13 years remission was reported by WINTROBE [20] but no mention was made as to the duration of the treatment.

Conventional drug treatment of CML generally fails to induce true remission since the bone marrow cells remain Ph¹-positive [8] Busulphan-induced bone marrow hypoplasia is frequently irreversible and even fatal, and patients who survive this phase usually do not remain in a long-lasting remission [10] GALTON and SPIERS [10] reported a few Ph¹-positive CML patients who survived a busulphan induced bone marrow hypoplasia and later on entered into an unusually prolonged remission lasting up to 14 years in these patients, the bone marrow hypoplasia was induced by relatively small doses of busulphan, and while in remission, their bone marrow became Ph¹-negative. It has been suggested by GALTON and SPIERS [10 cf 16] that in such patients the Ph¹-positive cells were unusually sensitive to busulphan and a marked reduction of the size of the Ph¹ positive population might be instrumental for the release of the normal Ph¹ negative cells from a homeostatic suppression caused by the mass of the proliferating Ph¹-positive cells [10] On the other hand, it may happen that the excessive amounts of busulphan administered to CML patients can eliminate the relatively small population of Ph¹ negative cells and thus be responsible for a fatal bone marrow hypoplasia The possibil

ity of deliberately inducing a 'planned' marrow hypoplasia, thus convert ing the bone marrow to a Ph -negative state has been considered by sev eral authors [4 9] although this procedure is not always successful [17] and not without harm [16]

The presence of Ph -chromosome, which is a major although not obligatory diagnostic finding for the establishment of the diagnosis of CML was searched for on several occasions in our patient, but was not detect ed. Since this examination was not performed when the diagnosis of CML was established, one may speculate that the bone marrow was actually Ph¹-positive at that lapse of time, and following therapy it became Ph¹ negative. This possibility seems unlikely since in previously described pa tients who were in prolonged remission, and converted into a Ph negative marrow some Ph¹-positive cells could always be detected during the course of their disease [6 16] Patients who have the less common form of Ph¹ negative CML, have generally a poor prognosis and a short survival [5 13] In this respect, the clinical course of our patient s disease is rather outstanding.

The excessive hypercalciuria detected prior to treatment was apparent ly associated with the bone lesions. Hypercalcemia and osteolytic lesions are found in 2.5–4.5 / of patients with acute leukemia [12, 19] but are relatively rare in the chronic forms of the disease [1] CHABNER *et al.* [2] reported 6 patients with CML in whom the initial manifestation of blastic transformation was the appearance of destructive bone lesions. STEINBERG *et al.* [18] reported another patient with CML in whom hypercalcemia associated with skeletal lesions closely preceded transformation into acute granulocytic leukemia. As observed in our patient, hypercalciuria may be present without hypercalcemia, this finding probably being due to a renal compensative mechanism of increased calcium excretion. Following treat ment, the hypercalciuria disappeared, eventually related to the decreased bone destruction.

Remission in CML patients with blastic transformation is achieved in only 20–45 of them [7 20] and, according to CANELLOS *et al.* [1] the medium survival was 10.5 months, whereas in patients resistant to treat ment it was 2.5 months. FOLEY *et al.* [7] suggested that following long-lasting cytotoxic treatment in patients with CML their bone marrow reserve is decreased. When blastic crisis occurs, they fail to enter into re mission because of a lack of bone marrow reserve. If this hypothesis is correct, it might be that the 12 / year-long interval without treatment, which preceded the blastic transformation in our patient played an impor

tant role in the achievement of the present remission. The question whether our patient has been 'cured' from the CML and after 13 years has succumbed to acute myelocytic leukemia remains open.

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F RUZICKA. **Elektronenmikroskopische Hämatologie.** Springer Wien 1976. XIII + 251 pp 145 fig. S 1090.-/DM 158.- ISBN 3-211-81391-8.

In diesem Buch behandelt der Autor nicht nur die elektronenmikroskopische Untersuchung von Blutzellen mit den herkömmlichen Methoden der Ultradünnschichttechnik, sondern auch die Oberflächenuntersuchung im Rasterelektronenmikroskop und die Zelldarstellung durch die Gefrierätztechnik. Nach einer sehr detaillierten Einführung in die Methodik werden die einzelnen hämopoetischen Zellen, die Blutzellen samt Thrombozyten und ihre Ultrastruktur systematisch beschrieben. Besonders ausführlich ist die Darstellung der abnormen Zellen bei den verschiedenen Leukämieformen und bei malignen Lymphomen. Neben den häufigen myeloischen und lymphatischen Leukämien werden auch seltene Bilder wie die Hairy Cells-Leukämie und das Sézary-Syndrom berücksichtigt. Die Abbildungen sind vortrefflich und die entsprechenden Legenden äusserst informativ Sowohl der klinisch wie der wissenschaftlich tätige Hämatologe oder Onkologe kann sich in diesem Werk über die faszinierenden morphologischen Aspekte und die praktische Bedeutung der zellulären Ultrastruktur bestens orientieren. P FRICK, *Zürich*

O PROKOP und W GÖHLER. **Die menschlichen Blutgruppen;** 4 neugestaltete Auflage. Fischer Stuttgart 1976. 290 pp., 37 fig., 73 tab. DM 29.- ISBN 3-437-10459-4

Die 4 Auflage des Bandes Die menschlichen Blutgruppen von PROKOP und GÖHLER bringt eine konzentrierte und klare Darstellung des aktuellen Wissens über die genetisch determinierten Marker des menschlichen Blutes. Die Autoren beschreiben die durch Antikörper definierten Erbmerkmale der Erythrozyten, Leukozyten und Thrombozyten, die erythrozytären Enzympolymorphismen sowie die Serummerkmale und geben auch, soweit bekannt, die chromosomale Lokalisation der korrespondierenden Genorte an. Abschliessend werden noch einige Probleme im Zusammenhang mit der Anwendung der Erbmerkmalsysteme des menschlichen Blutes für Paternitätsprobleme (Beweiswert von Vaterschaftsausschlüssen und Berechnung der Vaterschaftswahrscheinlichkeit) diskutiert.

Neben der theoretischen Besprechung der genetischen Situation innerhalb der einzelnen Systeme werden auch zahlreiche Details über die Bestimmungsmethoden der Bluterbmerkmale angeführt. Aus diesen Gründen ist der vorliegende Band für alle Leser die sich über die Genetik und die Definition der Erbmerkmale des menschlichen Blutes informieren wollen, wärmstens zu empfehlen.

W R. MAYR, *Wien*

M C. G ISRAELS and L W DELAMORE (ed.) **Haematological Aspects of Systemic Disease.** Saunders, Eastbourne 1976. X + 545 £ 17.50. ISBN 0-7216-5046-3

This book is an extension of the first volume of *Clinics in Haematology* the useful series which started in 1972. All the chapters have been brought up to date and

new chapters have been added. The first part, which gives concise account on the physiological bases of haematological disturbances on little more than 100 pages, should be particularly welcome to the physician who is not specialist in the field. He finds an excellent summary of the modern views on the various mechanisms underlying alterations in the blood. Whereas the chapter 'Red Cell' is rather too short, the ones on lymphocytes and platelets seemed particularly useful to the referee. Every chapter includes an extensive bibliography.

In the second part, secondary haematological changes in various systemic diseases are carefully outlined. There are particularly useful chapters on endocrine disorders, gastrointestinal disease or neurological disorders but also on malignant disease, skin disorders (where, funnily enough, eosinophilia is not mentioned), renal disease, rheumatoid arthritis and the connective tissue diseases, cardiology, infection, respiratory disease, geriatric medicine and liver disease. This well written book is concluded by a very fine chapter on drug-induced haematological abnormalities by R. H. Girdwood. On the whole, the two editors and the contributors, all well-known experts in their field, are to be congratulated for the achievement of this work, which definitely fills gap in the large number of haematological textbooks. It should be welcome to the specialist as well as to the general physician, to whom it is primarily directed. U. Buchler, *Bern*

C. Pochedly (ed.): **Leukemia and Lymphoma in the Nervous System.** Thomas, Springfield 1977. XIII + 229 pp., US $ 23.50. ISBN 0-398-03598-5

Increased frequency of central nervous system manifestations following successful control of peripheral disease has led t extensive clinical and experimental trials to better understand and treat this serious complication of leukemia and lymphoma.

CNS leukemia and lymphoma presents *itself in variety of clinical syndromes.* Its differential diagnosis is at times difficult and requires knowledge of infectious complications and neurotoxic side effects of drugs used in the treatment of leukemia and lymphoma. Great progress in the treatment of CNS leukemia and lymphoma has made this area of research an exciting and satisfying one.

It is therefore important to the physician treating patients with leukemia and lymphoma to have at hand book which guides into the aspects of CNS leukemia and lymphoma: pathophysiology, pathology, symptoms, diagnostic procedures with special reference to cytological techniques, neurotoxicity due to therapy – mainly intrathecal *chemotherapy and* CNS radiotherapy – treatment of the disease in great detail, prophylaxis and palliation. The book, well written, with abundant illustrations, figures and references, is highly recommended. G. A. Nagel, *Basel*

# Varia

## Ausschreibung des Georg-von-Hevesy Preises

Bei der Eröffnung des Weltkongresses für Nuklearmedizin am 17. September 1978 in Washington, D.C., wird der *Georg-von-Hevesy Preis* für Nuklearmedizin in Höhe von US$ 10 000 erneut verliehen. Der Preis gilt dem Gedenken an GEORG VON HEVESY dem Pionier der Nuklearmedizin, der 1943 mit dem Nobelpreis ausgezeichnet wurde.

Für den Preis können unveröffentlichte Arbeiten aus dem Gebiet der Nuklearmedizin eingereicht werden, vorzugsweise in Englisch, aber auch in Deutsch oder Französisch. Die Autoren dürfen das 45. Altersjahr am 17. September 1978 noch nicht vollendet haben. Das Manuskript darf 9 Maschinenseiten nicht überschreiten und muss bis zum 1. Juli 1978 bei Prof. Dr. W. HORST, Universitätsklinik für Nuklearmedizin, Rämistrasse 100, *CH-8091 Zürich* (Schweiz), eingetroffen sein.

Die *Georg-von-Hevesy-Medaille* wird am 24. Oktober 1978 anlässlich der Eröffnung des 16th International Annual Meetings der Gesellschaft für Nuklearmedizin (Europa) in Madrid an Frau ROSALYN S. YALOW, PhD verliehen, die 1977 den Nobelpreis für Medizin erhielt. Der Titel ihres Festvortrages, der Georg-von-Hevesy-«Memorial Lecture» lautet: Radioimmunoassay: Past, Present and Potential.

## Advances in Haematology

June 12–16, 1978

This Specialist Short Course is organized by the Royal Postgraduate Medical School, University of London. Applications are invited from Consultants and Senior Registrars. Topics include: Disorders of the red cells; Haemorrhagic disorders and thrombosis, and Leukaemia, lymphoma and allied disorders. Course organizers: Dr. S. M. LEWIS and Dr. S. M. WORLLEDGE. Course fee: £ 65 (including catering).

Application forms may be obtained from: The Deputy Secretary's Office (SSC), Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, *London W12 0HS* (England).

# Index rerum ad Vol. 59

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